Keeping cells healthy: using the chaperone network

A look inside the cellular toolbox

Lonneke Heldens

Voor mama

De helderste ster aan een donkere hemel

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Chapter 1

General introduction

1 Proteotoxic stress

Proteins make up a substantial proportion of the cell mass, and are involved in most regulated aspects of the activity in a cell. Multiple physiological or pathological conditions can affect proper protein folding, leading to accumulation of misfolded proteins which in turn leads to perturbation of the cell function, a process also known as proteotoxicity. Cells have developed various ways to cope with the diverse conditions that are encountered during a normal life span. These conditions vary from normal cellular growth and differentation to stressful pathophysiological states such as fever and inflammation [1]. The mechanism of the reaction to stress has been a continuous subject of research since the observations described by Ritossa in 1962 [2]. In this work a transient increase in temperature activated the expression of a small group of Drosophila genes encoding a set of proteins which became known as heat shock proteins (hsp's) [3]. Perhaps the name 'stress proteins' would have been more suitable since their synthesis is not only induced by heat but also by a wide range of other stressors, such as oxidative stress [4], hypoxia [5], viral infection [6], heavy metal contamination [7], ischemia [8], exercise [4] and metabolic stress [9]. The hsp encoding genes are highly conserved among species and their protein products can be assigned to families on the basis of conserved domains. In eukaryotic cells, these subfamilies comprise multiple members. These members differ in intracellular localization and function [10]. The products of stress-responsive genes are known to function in the protection and resistance to stress [11, 12]. During stress, the hsp's act as molecular chaperones to refold denatured proteins and to inhibit the accumulation of misfolded proteins, thereby helping the cell to endure the stress and survive. Also under normal conditions, hsp's serve to maintain proteostasis.

From the moment that the hsp's were discovered, the stress response leading to the synthesis of hsp's has been studied extensively and became known as the heat shock response (HSR). The HSR is a response designed to combat proteotoxic stress in the cytosol [13-15]. Now it is known that different intracellular compartments handle proteotoxic stress in a distinct way. The understanding of endoplasmic reticulum (ER) proteotoxicity has grown by characterization of the unfolded protein response (UPR) [16]. However, relatively less is known about protein homeostasis in other cellular compartments. Recently, the mitochondrial unfolded protein response has been described [17, 18].

2 Molecular Chaperones

Molecular chaperones can be classified into six major highly conserved families: Hsp100s (HSPH), Hsp90s (HSPC), Hsp70s (HSPA), Hsp60s (HSPD), Hsp40s (DNAJ), and small heat shock proteins (sHsp's, HSPB) [19]. In cells, a constant need for chaperone assistance is present. During de novo

protein folding and refolding of nonnative polypeptide chains chaperones are needed, since in the in vivo high protein concentration environment aggregation competes with productive folding. This is not only a problem during stress but even at physiological temperatures [19-21]. The three main cellular compartments, cytoplasm, mitochondria and ER have their own general chaperoning network. These networks are similar because they all contain related chaperones and associated factors which promote protein folding (Hsp90 and Hsp70 machines), chaperones which are known to deliver substrates to the folding machines (DNAJ/Hsp40 proteins) and chaperones which can store unfolded protein for later refolding or degradation (sHsp's). The main characteristics of the Hsp90, Hsp70, Hsp40 and sHsp family will be briefly discussed in this introduction because these families are, for the content of this thesis, the most relevant.

2.1 Hsp90 (HSPC)

Hsp90 proteins have three functional domains: the N-terminal, the middle domain involved in ATP hydrolysis and client and co-chaperone binding and a C-terminal dimerization domain [19]. The activity of Hsp90 is regulated by ATP binding and hydrolysis. Hsp90 family members have constitutive and stress related functions [22]. Hsp90 has many different client proteins such as steroid receptors, protein kinases, calmodulin, calcineurin, nitric oxide synthase (NOS), chloride channel CFTR, telomerase and transcription factors such as HSF-1, reviewed in [23, 24]. Over a dozen co-chaperones of Hsp90 are known in eukaryotes [25]. HSPC1 (HSP90AA1) is present in high amounts in the cytosol and it is further upregulated under stress conditions [26]. HSPC4 (HSP90B1, Grp94) is the ER Hsp90 paralog [27], while HSPC5 (TRAP1) is the mitochondrial Hsp90 paralog [28]. Hsp90 was shown to prevent the aggregation of the enzyme citrate synthase, yet the refolding of this enzyme requires the Hsp70 machinery as well, suggesting that there is cross-talk between both systems [29]. A similar co-operation between Hsp70 and Hsp90 has been described for maturation of steroid hormone receptors, reviewed in [30]. Co-chaperone STIP1 is unique because it interacts with both Hsp70 and Hsp90, providing a connection between the Hsp70 and Hsp90 chaperone machinery [31]. Hsp90 bound by ATP is stabilized by p23 [32], which replaces STIP1. CHIP modulates the balance between the folding of proteins and degradation of chaperone substrates [33].

2.2 Hsp70 (HSPA)

Hsp70 recognizes hydrophobic segments of unfolded polypeptides and binds them in an ATP dependent way. Hsp70 binds tightly to its substrates in the ADP-bound state allowing folding, whereas it associates and dissociates rapidly when ATP is bound. Hsp70 proteins contain an N-terminal ATPase domain and a C-terminal domain where the substrates bind. Between the ATPase domain and the C-terminal domain exists a small linker domain,

which couples the nucleotide hydrolysis to the ability of the C-terminal domain to bind substrate [34, 35]. Hsp70 family members are involved in de novo folding of newly synthesized proteins. During stress they are known to prevent the aggregation of unfolded proteins and they can refold unfolded or misfolded proteins in an ATP dependent way [36]. HSPA1A and HSPA1B are stress inducible members of the Hsp70 family located in the cytosolic compartment, whereas HSPA8 (Hsc70) is known to be constitutively expressed in the cytosol. HSPA6 is highly stress inducible, but lacks refolding capacities of heat-unfolded luciferase as well as the capacity to inhibit (polyQ) aggregation [37]. Nevertheless, HSPA6 is thought to be involved in cell survival after severe stress [38]. HSPA5 (also known as Grp78, BiP) is the ER Hsp70 paralog [27], while HSPA9 is located in the mitochondria [39]. The activity of Hsp70s is regulated by co-chaperones. Hsp40 proteins presents the substrate to the Hsp70 machine [40] and they accelerate ATP hydrolysis by Hsp70, which mediates strong binding of the substrate [41]. Additionally Hsp40 was shown to facilitate binding of the next component of the Hsp70 complex, the co-chaperone STIP1 (for review see [42]). STIP1 interacts with Hsp70 and Hsp90 simultaneously and transfers the substrate from the Hsp70 machine to the Hsp90 machine [43]. Bag-1 increases the ATPase activity of Hsp70 just like Hsp40 but Bag-1 inhibits release of the substrate, which results in inefficient folding [44], while ST13 prevents the dissociation of ADP and thus enhances refolding [45]. ST13 and Bag-1 compete directly for binding Hsp70. Interaction of CHIP with Hsp70 directs Hsp70 bound client proteins to the proteolytic pathway. However, the function of CHIP is thought to be more specific for the degradation of Hsp90 client proteins [33].

2.3 Hsp40 (DNAJ)

The largest class of Hsp70 co-chaperones are the Hsp40/ J-domain-containing proteins [35]. DNAJ family members are identified by the presence of a conserved J-domain. DNAJ family members are known to bind nonnative proteins and deliver these to Hsp70. The J-domains interact with the ATPase domain of Hsp70 and stimulate the hydrolysis of ATP. DNAJ family members can be subdivided in a type A, B and a type C. Type A and B contain an N-terminal J-domain, a glycine/phenylalanine-rich region, and a variable C-terminal domain. Type A proteins also contain a cysteine-rich region. Type C proteins only contain the J-domain which is not necessarily located at the N-terminus [46]. Only a few members of the DNAJ family are stress inducible, amongst which DNAJB1 and DNAJB9. The DNAJB family, in particular DNAJB1, is by far the most extensively studied DNAJ group. DNAJB1 cooperates with HSPA1A and HSPA8 in the cytosol and nucleus. DNAJA3 (Tid-1) isoforms exhibit a conserved mitochondrial DNAJ-like function [47]. DNAJB9 (ERdj4) [48] and DNAJB11 (ERdj3) [49] are ER specific and cooperate with HSPA5.

2.4 Small heat shock protein's (HSPB)

Ten sHsp family members have been described [50]. All of them share a protein sequence, which is generally referred to as the α-crystallin domain [51]. The sHsp family shows high heterogeneity in sequence but also in size [52]. Although sHsp's lack the refolding capacities of the large Hsp's, they can form large dynamic complexes composed of homo- or hetero-dimers. These homogeneous or heterogeneous oligomeric complexes are thought to be acting in an ATP-independent manner by interacting with large numbers of partially folded proteins to prevent interactions between hydrophobic domains of unfolded proteins [53, 54]. In this manner, aggregation of misfolded proteins is prevented. The substrate is kept in a folding competent state and can be transferred to the large Hsp's to be refolded [55] or to the proteasome which degrades the substrate [56]. The α-crystallin domain plays an important role in oligomerization as site-directed mutagenesis on HSP16.3 from Mycobacterium tuberculosis showed that particular residues on the α-crystallin domain are involved in the formation of β-sheets which are needed in oligomer formation [57].

A few HSPB's, e.g. HSPB1 and HSPB5, are induced by stress conditions. Human HSPB1 (hsp27) has three known phosphorylation sites (serines 15, 78 and 82) and becomes phosphorylated upon different stress stimuli like arsenite and heat. HSPB5 (αB- crystallin) can also be phosphorylated at Ser19, Ser45 and Ser59 upon different stress stimuli. Phosphorylation of HSPB1 monomers influences their oligomeric state [46]. Upon phosphorylation HSPB1 dissociates from large oligomers into small oligomers, presumably tetramers and dimers, to bind unfolded proteins [58, 59]. The dissociation into smaller oligomers leads to decreased in vitro chaperone activity of HSPB1 [60]. In vivo a HSPB1 mutant mimicking phosphorylation can protect the cells from the deleterious effect of a heat shock but not from other stresses, as oxidative stress [60-63]. Overexpressed pseudophosphorylated HSPB1 might also form mixed oligomers with endogenous HSPB proteins. HSPB4 and HSPB5 (αA- and αB- crystallin) are highly expressed in the eye lens were they provide a compact network of water-soluble proteins which is needed for the transparency of the lens. HSPB8 was shown to be involved in autophagy [64]. HSPB2 is located in the cytosol and mitochondria, no sHsp was found in the ER.

3 Transcriptional stress response cytosol

3.1 HSF family

The constitutively synthesized hsp's are expressed at a basal level and function under normal growth conditions. The stress-inducible forms of the hsp family are significantly induced following stress [65]. Expression of hsp's is under the control of a family of transcription factors which consists of seven

members: heat shock factor 1-5 (HSF1-5) [66, 67], HSFY1 [68] and HSFX1 [67]. The HSF family shows around 40% sequence homology within a species which is for the greater part limited to the oligomerization [69] and DNA-binding [70] domains.
HSF1 knock out models have unveiled that HSF1 is needed for the stress induced upregulation of hsp genes, and to gain thermotolerance (discussed below) [71-74]. HSF2 is required for oogenesis, spermatogenesis and brain development [75]. During development HSF2 becomes highly expressed leading to spontaneous activation of HSF2 [76]. HSF3 has been found in avian species, were it regulates cellular development and the stress response [77, 78]. Recently a murine HSF3 was found. This protein translocates to the nucleus upon heat shock but was not able to activate classical hsp genes [79]. HSF3 is absent in human. The fourth family member, HSF4, has two isoforms namely HSF4a and HSF4b, as a result of alternative splicing [80]. Both isoforms lack the heptad repeat (HR)-C which is needed to suppress HSF trimer formation. HSF4a has been thought to be a negative regulator of the heat shock response and the stress-induced gene expression [81], while HSF4b acts as a transcriptional activator [80]. Furthermore, HSF4 was shown to be needed for the development of the lens and lens fiber cell differentiation [82]. HSF5 and HSFX1 are not predicted to bind the heat shock element and are of unknown function [67]. HSFY1 has been mapped on the Y chromosome [68] and altered expression of HSFY1 in the testis results in deteriorated spermatogenesis [83].

3.2 HSF1 structure

A model of the functional domains of HSF1 is shown in figure 1. HSF1 contains a DNA-binding domain, hydrophobic repeats which are necessary for trimerization, a regulatory domain and an activation domain located at the C-terminal part of the protein. The DNA binding domain, located near the N-terminus of HSF1 is composed of a helix-turn-helix motif [84]. Trimerization of HSF1 occurs via a classical helical coiled-coil interaction of HR-A/B and HR-C domains [85]. HR-C associates with the HR-A/B in unstressed cells to form an intramolecular coiled coil. In this particular conformation, trimer formation becomes repressed [85, 86]. When HSF1 becomes activated, the intramolecular interactions become disrupted and intermolecular interactions are formed through HR-A/B to form an HSF1 trimer [69, 87]. The trimeric form of HSF1 possesses an increased affinity for the heat shock element (HSE) [88]. The HSE is located upstream of hsp genes and is composed of multiple elements containing three or more pentameric nucleotide repeats of 5'-nGAAn-3' that vary in orientation. Each DNA domain of the HSF trimer binds a GAA [89-91]. The transactivation domain of human HSF1 is located at the C-terminal part [66] and can be dissected into two distinct activation domains AD1 and AD2 which together ensure a fast and prolonged response upon stress [92]. A negative regulatory domain is located between the HR-A/B and HR-C domains. This domain causes repression of the transcription-

al activation domain of HSF1 in unstressed cells [92, 93].

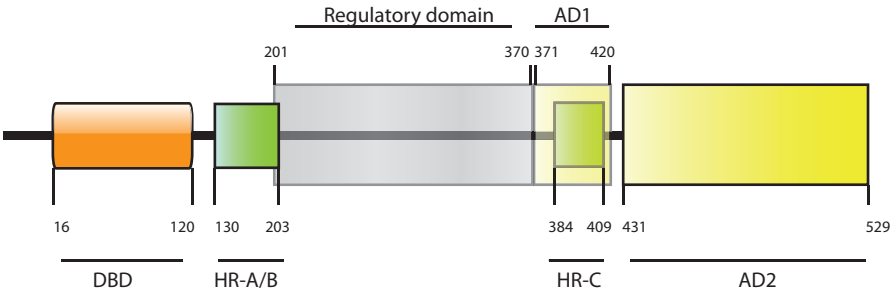


Figure 1 Organization of HSF1 protein.
Schematic representation of human HSF1. DNA binding domain (DBD), hydrophobic repeats (HR-A/B), the regulatory domain, hydrophobic repeat (HR-C), and the AD1 and AD2 transactivation domains are shown. This scheme has been adapted from [66, 92-95].

3.3 Regulation of HSF1 and kinetics of HSR

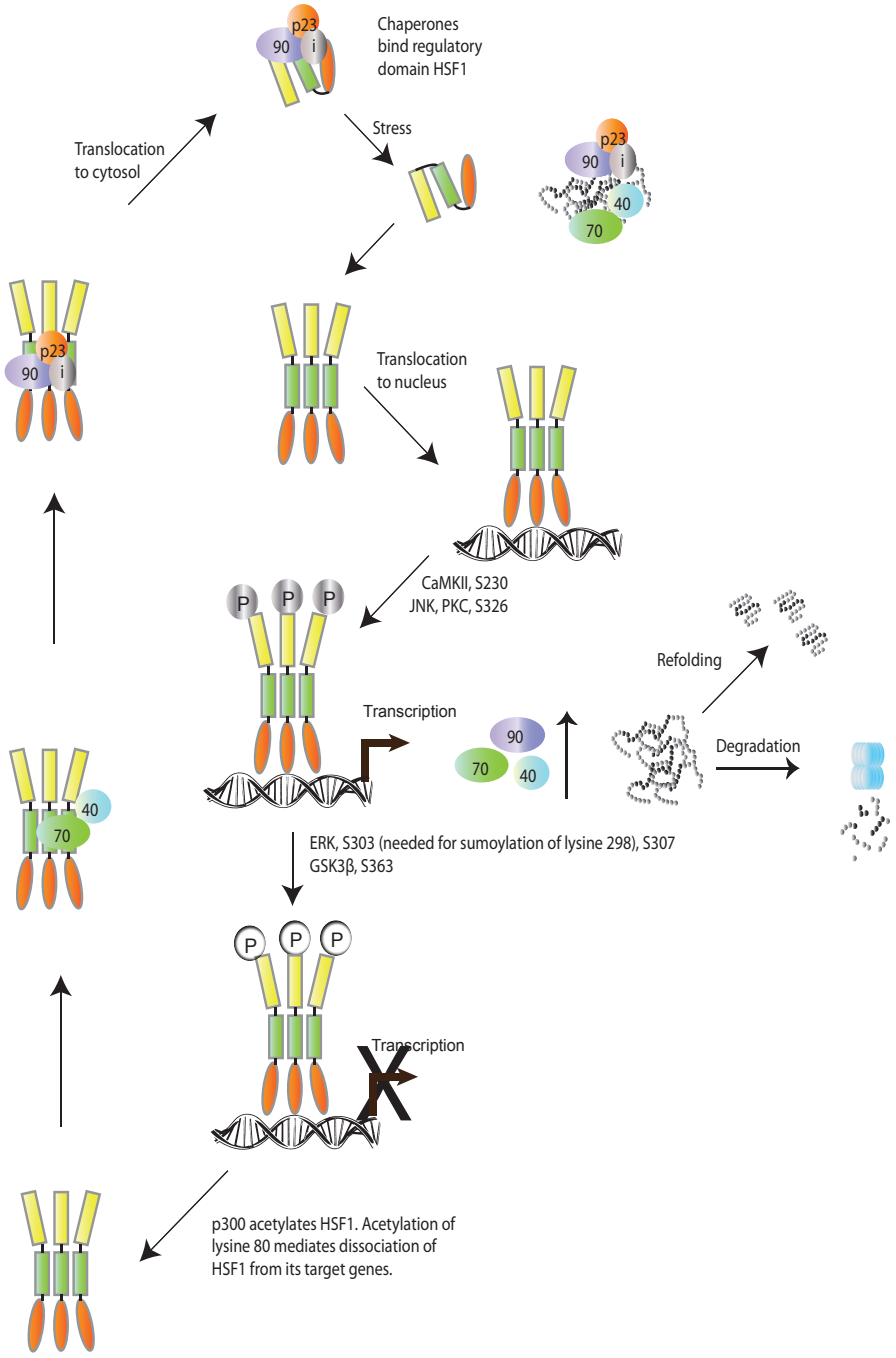
Under non stress conditions, HSF1 is found in a multichaperone complex located in the cytoplasm. The signal triggering activation of the HSR is the accumulation of nonnative proteins in the cytosol, as a result of chemical or physical denaturation [96-102]. The total concentration of client proteins for chaperones increases and competes with HSF1 for binding, as a result the concentration of chaperone bound HSF1 will decrease. Free HSF1 homotrimerizes, subsequently, trimeric HSF1 translocates to the nucleus binding DNA at the highly conserved HSE sequences located in the promoter regions of hsp genes [103] (Fig. 2). When transcriptionally competent HSF1 transactivates chaperone genes, the concentration of chaperones increases. The newly synthesized chaperones accelerate removal of denatured proteins and promote refolding, thus the concentration of client proteins for chaperones decreases as the amount of chaperones increases and the amount of nonnative proteins decreases [104]. Finally HSF1 adopts its unactive state and reassembles with the chaperone complex. The reassembly of the chaperone complex with HSF1 monomers is one of the last HSF1 inactivation steps. Early reports suggested that HSF1 is regulated by the amount of free Hsp70 [105, 106]. Others showed a role for Hsp90 as being a key regulator of HSF1 activity. Here, HSF1 is maintained in a monomeric form in the cytoplasm via a heterocomplex that involves Hsp90 [107, 108], p23 [109], as well as immunophilin [110] but other chaperones are probably also involved.

Compounds as such salicylates and hydrogen peroxide induce HSE DNA-binding activity but are not able to induce expression of the inducible hsp genes [111, 112]. In addition, heat exposure of an erythroleukemia cell line results in HSE DNA-binding activity but no increase in chaperone expression was seen [113]. These results imply that HSF1 activity is regulated

at multiple levels, DNA binding of HSF1 is not sufficient for transcriptional activation, HSF1 also needs to be activated in another way. Therefore HSF1 must be targeted by other regulatory mechanisms as well. HSF1 is post-translationally modified (see below) and associates with other transcription factors such as HSF2, STAT-1 and NF-IL6. Although HSF1 is known as the master regulator of the transcriptional response upon stress, HSF2 was also shown to bind promoters of hsp genes during stress. This binding activity of HSF2 is dependent on the presence of HSF1 [114]. During stress transcriptional competent HSF1-HSF2 heterotrimers are formed. Prolonged stress decreases the HSF2 levels, thereby limiting heterotrimerization. It has been postulated that HSF1 mediated transactivation might be modulated by HSF2 levels [115]. HSF1 and HSF2 heterotrimerization might provide a switch between transcriptional activation in response to diverse stimuli. HSF1 was also shown to associate with regulatory factors like DAXX, splicing factors, chromatin remodeling factors and components of the transcriptional machinery (reviewed in [116]).

HSF1 is a constitutively phosphorylated protein which under stress conditions is inducibly hyperphosphorylated [86, 117, 118]. Phosphorylation induced by stress results in modification of 12, and maybe more, serine residues [119]. Phosphorylation of Serine -303, -307, and -363 were shown to have negative regulatory effects [117, 120, 121]. Under normal conditions, these serine residues are constitutively phosphorylated; the phosphorylation state is mediated by multiple kinases [117, 122]. ERK was shown to phosphorylate HSF1 at serine 303 and serine 307 was shown to be phosphorylated by GSK3β [120, 123]. Serine 363, which is located adjacent to AD1, was found to be phosphorylated by JNK and PKC [120, 124]. Two positive regulatory sites have been found within the HSF1 protein. Serine 230 is phosphorylated under normal conditions, but undergoes enhanced phosphorylation under stress conditions by the calcium dependent protein kinase II (CaMKII) [125]. Furthermore, phosphorylation of HSF1 at Serine 326 was shown to induce transcriptional enhancement [119]. Phosphorylation of none of the known sites is absolutely required for activation of HSFl in response to a stress or subsequent inactivation after stress. Phosphorylation of Serine 303 is needed for SUMOylation of HSF1 at Lys298 [126, 127]. SUMOylation does not affect the DNA binding ability of HSF1 [128], but the transactivational capacity of HSF1 becomes impaired when HSF1 is SUMOylated [128, 129].

Figure 2 Activation of HSF1.
Under normal conditions, HSF1 is a monomer which is kept inactive by binding of chaperones. During stress, the chaperone complex dissociates from HSF1, allowing HSF1 to from trimers and to bind heat shock elements located in the promoter regions of heat shock protein genes. Then the phosphorylation events start with transcription enhancing phosphorylation at HSF1 residues S230 and S326 and they end with repressive phosphorylation at S303, S307 and S363, followed by SUMOylation and acetylation, which also suppress HSF1 activity. Finally HSF1 reassembles with the chaperone complex.


p23
90
i
Chaperones
bind regulatory
domain HSF1
Stress
Translocation
to cytosol
40
70
Translocation
to nucleus
CaMKII, S230
JNK, PKC, S326
P
Transcription
Refolding
Degradation
ERK, S303 (needed for sumoylation of lysine 298), S307
GSK3β, S363
Transcription
p300 acetylates HSF1. Acetylation of
lysine 80 mediates dissociation of
HSF1 from its target genes.

Phosphorylation of serine sites which repress stress induced transcriptional activation of HSF1, and SUMOylation, which also represses transcriptional activity of HSF1, are relatively early events in the stress response. HSF1 is suggested to be acetylated by histone acetyltransferase p300, and acetylation triggers the dissociation of HSF1 from its target genes. HSF1 is acetylated on at least nine lysine residues. Acetylation of Lys80 controls HSF1 binding to DNA [130]. SIRT1 directly deacetylates HSF1 and thereby regulates the heat shock response. Decreasing HSF1 acetylation has been implicated to prolong HSF1 binding to target promoters, and to enhance the heat shock response.

In summary, the present model is not complete enough to explain all (HSF1 dependent) events during and after stress. The time that HSF1 is bound to the DNA is cell type dependent and depends on the dose of stress [130, 131]. The transient nature of HSF1 binding makes explaining the HSF1 dependent induction of chaperones at a later time point after stress difficult. For example, HSPA1A protein levels increase relatively early during the HSR, while HSPB1 protein levels increase at a later time point, when HSF1 is not thought to be bound or transcriptionally active anymore. The HSPA1A promoter is the most wide spread model used to monitor transcriptional activation of HSF1. This model might be, to some extent, limited because the stress response appears to have more phases and this is also not being monitored. Furthermore, the interplay between the different HSFs is not included. This suggests that using the HSPA1A promoter as a model system is not enough to unveil the complete picture regarding the transcriptional activation of HSF1.

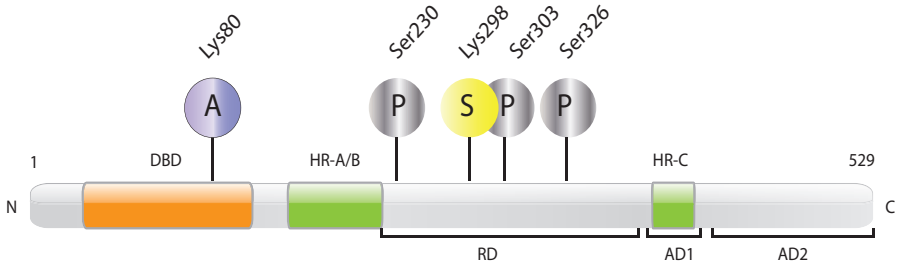


Figure 3 HSF1 becomes post-translationally modified upon proteotoxic stress.
The transactivational activity of HSF1 is regulated by several post translational modifications, such as phosphorylation and SUMOyaltion. During the attenuation phase of the stress response, Hsp's represses the transactivation activity of DNA bound HSF1 and DNA binding is inhibited by acetylation of Lysine 80 located in the DBD of HSF1 [132].

4 Translational control during stress

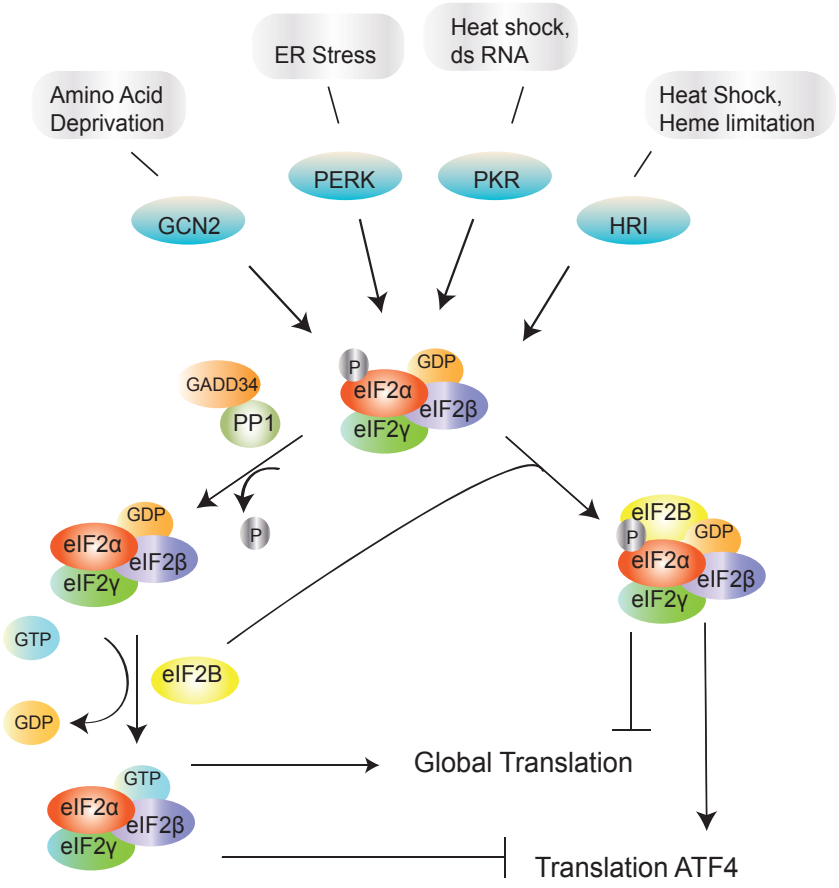
4.1 Stress induced leaky scanning

Translation initiation involves the assembly of the ribosomal 40S subunit, several initiation factors and a ternary complex (eIF2 bound to GTP and the initiator $tRNA_i^{Met}$) at the 5' cap of the mRNA (reviewed in [133]). This assembly, together called the 43S complex, scans the mRNA for the initiation codon. Before the 60S subunit can bind, eIF2-GTP is hydrolyzed to eIF2-GDP. eIF2 is recycled to an eIF2-GTP bound form in a reaction catalyzed by eIF2B. eIF2 is a complex of three polypeptide chains, α-, β- and γ-. eIF2α is the major target for translation regulation under various stress conditions including heat shock, accumulation of unfolded or denatured proteins in cytosol or ER lumen, changes in intracellular calcium, nutrient deprivation, virus infection or the induction of apoptosis. Different kinases mediate the phosphorylation of eIF2α: GCN2 (amino acid starvation, UV), PKR (dsRNA or heat), HRI (low heme, arsenite, osmotic stress, heat stress), and PERK (ER stress). When phosphorylated on Serine 51, eIF2α binds to eIF2B and prevents the exchange of GDP to GTP, thus reducing the availability of the ternary complex which is needed for translation initiation. When the availability of the ternary complex is low, initiation of translation of messengers is largely inhibited. Phosphorylation of eIF2α upon stress thus results in the inhibition of general protein synthesis (Fig. 4a). Paradoxically, it can also result in the translation of downstream ORFs (Fig. 4b). Around 10% of the eukaryotic mRNAs contain upstream open reading frames (uORFs) [134]. Well known messengers containing a uORF are ATF4, ATF5, CHOP and GADD34 mRNAs.

The uORFs normally inhibit translation of the downstream ORFs, but leaky scanning and reinitiation make the translation of the downstream ORF possible [135]. After translation of the fisrt uORF, ribosomes resume scanning in the 5' to 3' direction. Under non- stress conditions, $eIF2/GTP/tRNA_i^{met}$ is plenty available. The small ribosomal subunit will bind $eIF2/GTP/tRNA_i^{met}$ and couples with the 60S ribosome. Then reinitiation of translation occurs and the second ORF will be translated. In the case of ATF4 messenger, the second ORF overlaps the third ORF (which encodes ATF4) partially and is inhibitory because it is out of frame with the ATF4 ORF. After translation of uORF2, ribosomes will dissociate from the mRNA without translating ATF4 ORF. When eIF2α is phosphorylated, the levels of $eIF2/GTP/tRNA_i^{met}$ will be reduced. After translation of uORF1, the ribosomes have more scanning time before they will be recharged with $eIF2/GTP/tRNA_i^{met}$. The chance that the ribosome skips the AUG of the second ORF and reinitiates at the ATF4 ORF increases, which results in increased translation of the ATF4 ORF [136] (Fig. 4b). ATF4 was shown to be a strong transcriptional activator [137-141]. Some of the genes induced by ATF4 are CHOP (GADD153) [142], GADD34

[143] and asparagine synthetase [144, 145].

4 A eIF2α-phosphorylation.



4 B ATF4 translation.

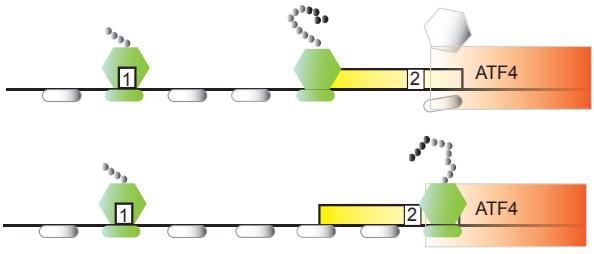


Figure 4 Translational control stress response.
a) A wide spectrum of cell stress signals are transduced by four eIF2α kinases; GCN2, double-stranded RNA-activated protein kinase (PKR), double-stranded RNA activated protein kinase-like ER kinase (PERK), and heme regulated inhibitor kinase (HRI). All four kinases phosphorylate eIF2α on serine 51. eIF2α-P binds eIF2B in a non-functional complex which leads to suppression of global translation and a paradoxical increase in translation of some ORFs, such as that encoding ATF4. Dephosphorylation of eIF2α is mediated by protein phosphatase 1 (PP1), which is targeted to eIF2α by growth arrest and DNA damage-inducible 34 (GADD34). Adapted from [146].
b) Synthesis of ATF4 is increased in response to cell stress induced eIF2α phosphorylation. The mRNA for ATF4 includes two short upstream open reading frames (uORF). In non stress conditions, ribosomal scanning leads to translation of uORF1 and re-initiation at uORF2, which is out-of-frame and has an overlap with the ATF4 coding sequence leading to a low rate of ATF4 synthesis. During stress, eIF2α becomes phosphorylated which causes a depletion of the available GDP-GTP exchange factor, eIF2B, by non-functional binding. The amount of active eIF2-GTP is reduced and results in a slower rate of re-initiation after uORF1 translation. Consequently, ribosome assembly occurs after the AUG in uORF2 with translation initiation at the ORF encoding ATF4 [136].

5 The Unfolded Protein Response

The ER is the site where secretory and membrane proteins are synthesized. Proteins folded by chaperones inside the ER are transported to the Golgi apparatus. Unfolded or misfolded proteins are kept inside the ER and ultimately degraded by ER-associated degradation (ERAD). ERAD targets selected by a quality control system within the ER lumen are degraded by the cytoplasmic ubiquitin proteasome system [147, 148]. When unfolded proteins accumulate inside the ER, the UPR becomes activated. Just like the HSR, the UPR induces transient attenuation of protein synthesis and transcriptional induction of genes to expand the protein-folding and protein-degradative capacities of the ER [16, 149]. The UPR is regulated by three ER-localized transmembrane proteins: inositol requiring 1α (IRE1 α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). The ER chaperone protein HSPA5 (BiP) serves as a master UPR regulator. It associates with, and represses activity of IRE1α, PERK and ATF6. Upon accumulation of unfolded proteins in the ER, the unfolded proteins bind HSPA5 and sequester it from interacting with IRE1α, PERK and ATF6 to elicit activation of these three ER membrane proteins [150].

5.1 IRE1α

Upon activation of the UPR, splicing initiated by IRE1α removes a 26-nucleotide intron from unspliced mammalian X-box binding protein 1 (XBP1) mRNA, to induce a translational frameshift producing a fusion protein encoded from two evolutionarily conserved open reading frames [151]. XBP1 is a basic leucine zipper type transcription factor involved in the UPR. It was reported that the unconventional splicing occurs in the cytoplasm without

nuclear processing when the catalytic domain of IRE1α is ectopically expressed in the cytoplasm [152, 153]. The catalytic domain of IRE1α contains a nuclear exclusion signal to prevent mislocalization of IRE1α to the nucleus which indicates that the splicing of XBP1 mRNA occurs predominantly in the cytoplasm [154].

XBP1s activates transcription of ER chaperone and ERAD genes as a homodimer or a heterodimer with ATF6, whereas XBP1u, encoded by unspliced XBP1 mRNA, enhances degradation of XBP1s [155, 156]. The promoter of XBP1 carries an ERSE where ATF6 can bind to activate transcription. The increase in XBP1 mRNA levels was greatly reduced in Chinese hamster ovary (M19) cells lacking Site-2 protease, which is important for the activation of ATF6. Cells lacking Site-2 protease were more sensitive to ER stress than wild type cells, but this effect could be reversed by overexpressing XBP1 [157]. In some organs and cells, XBP1s is required for protein secretion. In plasma cells XBP1s is necessary to produce immunoglobulin and in pancreatic exocrine cells it is needed to produce zymogens [158, 159]. In adult liver XBP1s does not affect protein secretory function but rather controls growth and survival of hepatocytes, thereby influencing processes such as lipogenesis [160, 161]. There are conflicting reports about the role of XBP1s in cellular integrity. XBP1s has been implicated to positively influence cell survival. XBP1s overexpression protected fibroblasts against cell death induced by ER stress [162]. Furthermore, the exogenous expression of XBP1s had protective effects against proteasome inhibitor induced dopaminergic cell death [163]. In addition, more cell death occurred in hydrogen peroxide treated XBP1 deficient cells compared with wild type cells. Recently, IL-3 signaling in murine pro-B-cells cells was shown to enhance XBP1 promoter activity and to promote XBP1 splicing. Inhibition of IL-3 signaling or knockdown of XBP1 induces apoptosis, while constitutive expression of XBP1s protects BaF3 cells from apoptosis during IL-3 depletion [164].There are also reports suggesting a negative role for XBP1s in cell survival. Mutations in SOD1 cause familial amyotropic lateral sclerosis. Mice harboring mutant SOD1 but lacking XBP1 were more resistant to developing disease compared with mice having mutant SOD1 and no XBP1 deficiency, possibly because increased levels of autophagy in motor neurons [165]. Overexpression of XBP1s induced apoptosis in HUVECs by activating caspase and downregulation of VE-cadherin [166]. Furthermore, XBP1s overexpression impaired glucose stimulated insulin secretion in rat beta cells and increased levels of beta cell apoptosis were observed [162]. These reports stress that the role of XBP1s is condition and cell type dependent. Therefore expression of XBP1s is tightly regulated.

IRE1α targets several RNAs other than XBP1 mRNA. The function of the cleavage of these targets under stressed conditions is not clear [167]. IRE1α has also signaling functions beyond its nucleolytic property: phosphorylated IRE1α can also recruit tumor necrosis factor receptor associated factor 2 (TRAF2). IRE1α and TRAF2 interact with apoptosis signal-regulating kinase

1 (ASK1), which phosphorylates and activates JNK [168, 169]. TRAF2 also interacts with procaspase-12 and promotes the clustering of procaspase-12 and its activation by cleavage in response to ER stress [170]. JNK also phosphorylates the anti-apoptotic protein BCL-2 at the ER membrane. This could affect the initiation of macro-autophagy because phosphorylated BCL-2 dissociates from Beclin-1 and this dissociation induces activation of Beclin-1 [171], an essential component of the macro-autophagy machinery [172]. It has also been shown that knocking down UPR components in Drosophila melanogaster results in increased basal macro-autophagy levels, suggesting a link between the UPR and macro-autophagy [173].

5.2 ATF6

ATF6 is an ER transmembrane transcription factor with the C-terminus located in the ER lumen and its N-terminal DNA binding domain faces the cytosol [174, 175]. ATF6 is thought to be associated with HSPA5 and is released from HSPA5 upon ER stress. Two isoforms of ATF6 are known, ATF6α (90 kDa) and ATF6β (110 kDa). The ATF6α homolog is thought to be responsible for transcriptional regulation of prosurvival genes during ER stress, but ATF6β may also play a role. Upon activation of the UPR, ATF6α and ATF6β translocate to the Golgi where they are cleaved by site-1 protease and site-2 protease to generate 50-kDa cytosolic b-ZIP-containing fragments that migrate into the nucleus. In the nucleus transcription of UPR target genes is activated [176, 177]. ATF6 was shown to bind to the ER stress response element (ERSE). ERSE is a short consensus sequence found in the promoter region of UPR target genes [178] such as CHOP (a gene implicated in apoptosis), and XBP1 [179]. ATF6 induces transcription of ER chaperone genes as well as ERAD genes.

5.3 PERK

Activation of the eIF2α kinase PKR-like endoplasmic reticulum kinase (PERK) is part of the UPR [136]. As described earlier, eIF2α-P results in a decrease in the global rates of translation initiation and is one of the earliest events of the UPR. This will not only reduce the load of protein folding on the ER, but will also causes the release of ribosomes and translation factors from the mRNA. This resetting of the translational programme is proposed to help newly synthesized mRNAs that are transcribed by the UPR induced gene-expression programme to compete for limiting translation factors [136]. Furthermore, ATF4, GADD34, CHOP [180, 181] and ATF5 ORFs are translated [182, 183]. The induction of chaperone-encoding genes by the ATF6 and IRE1α pathways seems to be at odds with the repression of mRNA translation. However, GADD34, a target of ATF4, mediates eIF2α dephosphorylation and serves as a feedback mechanism. GADD34 coordinates the recovery of eIF2B activity and thereby enhances translation initiation and with the transcriptional induction of UPR target genes, promotes the translation of their mRNAs [184, 185].

5.4 ERAD

The process Endoplasmic reticulum-associated degradation (ERAD) mediates translocation of proteins from the ER to the cytosol. In the cytosol the proteins are degraded by the 26S proteasome, a multicatalytic protease which is enriched at the membrame of the ER and degrades ubiquitinated polypeptides [186-190]. ERAD removes potentially dangerous proteins from the secretory pathway when the concentration of misfolded proteins accumulates to high levels [191, 192]. Proteins to be degraded are ubiquitinated. Different ubiquitin ligase complexes are used to tag each class of protein (misfolded lumenal, misfolded transmembrane and proteins with misfolded cytosolic domains), suggesting that the ERAD pathways differ for varying classes of proteins [193, 194].

5.5 ER stress induced apoptosis

The UPR does not only include prosurvival pathways, but can also activate apoptosis cascades. The IRE1α pathway elicits apoptosis via activation of JNK. Activation of the PERK and ATF6 pathways lead to transcriptional induction of CHOP, which was shown to have pro-apoptotic properties [195]. Chop−/− cells are protected from ER stress-induced apoptosis [196]. The precise mechanism by which CHOP mediates apoptosis in unclear, CHOP activates the transcription of several genes that could trigger apoptosis. These include GADD34, ERO1, DR5 and TRB3 [149].

5.6 Kinetics of UPR signaling

The IRE1α, ATF6 and PERK pathways of the UPR do not operate simultaneously [197]. The first immediate response involves the PERK pathway to decrease the load of unfolded proteins in the ER by attenuating translation. Additionally the ATF4 ORF becomes translated. If unfolded proteins still accumulate, the ATF6 and IRE1α pathways increase the expression of ER chaperones to enhance the folding of unfolded proteins and to increase the clearance of misfolded or unfolded proteins by upregulating the capacity of ERAD. Macro-autophagy is also activated by the IRE1α pathway, to remove aggregated proteins. ATF6 activation is accomplished by cleavage of a preexisting transmembrane protein, while XBP1 needs to be transcribed, spliced and translated before it is an active transcription factor. Therefore detection of active ATF6 precedes detection of XBP1s in ER stressed cells [151]. ATF6 prefers NF-Y-dependent ERSE binding to NF-Y-independent UPRE binding, while XBP1s binds both ERSE and UPRE [151]. Target genes under the control of ERSE are ER chaperones such as HSPA5 and GRP94, while the promoter regions of components of ERAD like EDEM contain an UPRE. XBP1s is able to transactivate its own transcription, allowing the activity of XBP1 to continue as long as IRE1α is activated [198]. These findings suggest that cells activate ATF6 to induce transcription via ERSE directly in response to ER stress, prior to activation of XBP1s to induce not

only ERSE-mediated but also UPRE-mediated transcription. During prolonged ER stress, the IRE1α pathway and the ATF6 pathway are turned off, while the PERK pathway remains active. This shift results in a transition from adaptation/survival to pro-apoptotic conditions [199].

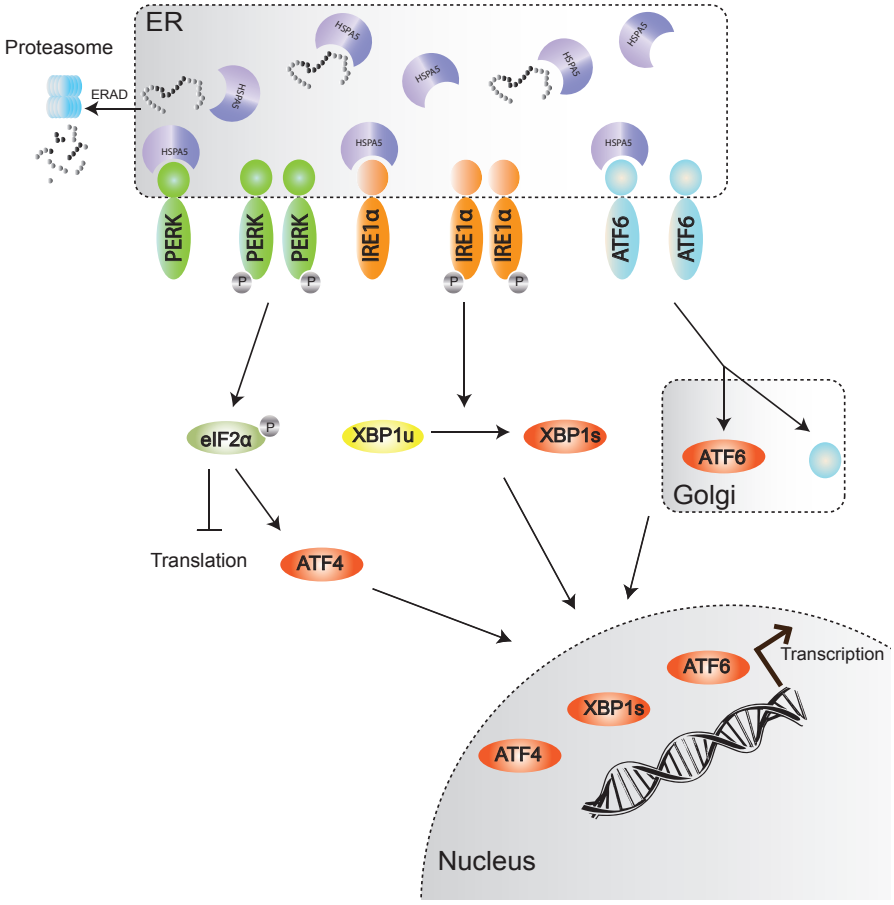


Figure 5 Unfolded Protein Response.
Upon aggregation of unfolded proteins, HSPA5 dissociates from the three ER membrane receptors, PKR-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6). Dissociation from these receptors leads to activation of the three pathways. The three pathways co-operate to restore ER function by inhibiting the production of new client proteins, increasing the folding capacity and promoting degradation of protein aggregates.

6 The physiological importance of cellular proteostasis

6.1 Role of HSF1 in the absence of stress

HSF1 is not only important during stress, various reports suggest a transcription regulatory role for HSF1 in de absence of stress. HSF1 null mice show the expected stress-related phenotypes, such as a complete lack of the heat shock response and the inability to develop thermotolerance (discussed below). However, they also suffer from neuronal, developmental and germ cell defects [74, 200-203], which cannot be directly linked to the heat shock response and which strongly suggests that HSF1 also regulates gene expression under non-stress conditions. Microarray analysis resulted in the identification of 49 genes (19 related to immune response) that are expressed at reduced levels in HSF1 null fibroblasts compared with wild type cells cultured under physiological conditions. The immune response of HSF1 null mice was shown to be severely impaired [204]. More recently, direct evidence for the stress independent regulation of genes by HSF1 was provided in the case of the multi-drug resistance gene 1 [205], and the IL-6 gene [206]. Furthermore HSF1 inhibits heregulin induced transcription in breast carcinoma cells [207]. It has also been discovered that mice carrying a mutant HSF1 gene show altered circadian period lengths, suggesting that HSF1 is of functional importance for the circadian clock [208].

6.2 Thermotolerance

Prior exposure to a single preconditioning stress allows cells [209], tissues [210], and animals [211] to survive an otherwise lethal stress dose. This phenomenom is called thermotolerance and was first described by Gerner et.al. [212], who noticed that cellular sensitivity to a second hyperthermic dose was reduced after an initial thermal dose followed by a recovery period at 37 °C. Additionally, a stronger initial dose induces a longer transient state of thermotolerance and makes that a cell can survive stronger stresses.
To acquire the state of thermotolerance, the recovery period at 37 °C was shown to be crucial. The upregulation of the synthesis of several chaperones closely correlated with the level of thermtolerance [213]. The synthesis of these chaperones is not only upregulated upon heat stress, but also upon other types of stresses. Furthermore, overexpression of various chaperones confers tolerance to a subsequent stress [214] and inhibition of the synthesis of chaperones by using blocking antibodies decreases the survival rate [215]. Overexpression of HSPA1A increases ER stress-induced activation of the IRE1α axis and promotes survival during ER stress. HSPA1A was shown to bind and enhance the RNase activity of IRE1α. This provides a molecular link between the heat shock response and the ER stress response [216].

6.3 The critical node of the chaperone network strongly depends on the substrate which is used for measuring

Cells have an extensive chaperone network to maintain protein homeostasis. To identify the critical nodes in this network various approaches can be used. Polyglutamine (polyQ) diseases are caused by an expansion of a triplet repeat in the causative gene and misfolding and aggregation are both associated with the cytotoxicity in polyglutamine diseases. Molecular chaperones have been shown to ameliorate disease progression. Measuring aggregation of polyQ proteins gives an indication of the chaperone capacity of a cell. Depleting cells of chaperones by overexpressing a dominant negative HSF1 mutant promotes aggregation of polyQ proteins. A subfamily of the DNAJB family was shown to be able to suppresses polyglutamine aggregation and associated toxicity both in mam¬malian cell-lines as well as in an in vivo Xenopus model [217]. The sHsp's HSPB1 and HSPB8 are also able to inhibit the aggregation of polyQ proteins.
Chaperone activity can also be monitored by measuring refolding of heat denatured luciferase 'in vivo'. Cells are transfected with a luciferase expression construct. Heat shocking the cells causes luciferase to denature which leads to loss of activity. Endogenous chaperones are able to refold luciferase and thereby recovering the activity. Introducing an excess of chaperones protects luciferase from unfolding and also enhances refolding after denaturation; depleting cells of chaperones has the opposite effect. Refolding of luciferase can be increased by exogenous expression of HSPA1A and is mainly dependent on the Hsp70 machinery.
Another tool to monitor chaperone activity is the glucocorticoid response. Efficient maturation of the glucocorticoid receptor (GR) to a conformation which has a high affinity for hormone binding requires the Hsp70 and the Hsp90 machines (for review see in [218]). Whereas the Hsp70 mediates folding of the GR, the Hsp90 machine is needed for the maturation of the GR [219, 220]. Members of the DNAJB family were able to rescue an impaired glucocorticoid response as a result of chaperone depletion. Which node of the cytoplasmic chaperoning network is critical thus depends on the substrate tested.

7 Aging

Aging is a multifactorial and complex process that involves the gradual impairment of normal biological cellular functions and which results in increased sensitivity to stress and a decreased ability to survive [221]. One of the hallmarks of aging is the increase in aberrant proteins which are prone to aggregation. Furthermore, a strong decline in degradation of proteins occurs [222], as the proteosomal function declines with age [223, 224]. In addition, lysosomes, where intra-cellular proteolysis can be achieved, become

significantly affected with age (due to accumulation of lipofuscin, a non-degradable intralysosomal polymeric aggregate) [225]. Lysosomal degradation of dysfunctional intracellular components can occur via three different pathways; macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). The activity of CMA was shown to decline with age [226]. Therefore, unfolded proteins tend to accumulate within the cell with age.

7.1 The HSR in aging

During aging, the DNA binding capacity of HSF1 becomes impaired which results in reduced expression of heat shock proteins [227, 228]. Furthermore, abnormal increases in GSK3β levels have been shown in Alzheimer's disease, which leads to inhibition of HSF1 activity [229]. An age related reduction of the HSR was not only found in neuronal tissue but also in skeletal and cardiac muscle [230] and in liver tissue [231]. The loss of functional protein or the presence of misfolded proteins can result in a wide range of diseases involving deposition of aggregated proteins, which includes Alzheimer disease, Parkinson disease, Huntington disease, and spongiformen-cephalopathies (prion diseases). Dysfunction of the HSR plays an important role in these neurodegenerative diseases [232-234].

Other studies which implicate a role for HSF1 in the aging process have shown that increased HSF1 activity is positively related to the lifespan of nematodes and flies, whereas decreased expression of HSF1 decreases the lifespan of C.elegans [235, 236]. Hsp70 and shsp's were shown to play a role in the lifespan of Drosophila [237, 238].

In C. elegans and Drosophila it has been demonstrated that decreased activity of the insulin/IGF1 pathway leads to an extended lifespan [239, 240]. This effect requires HSF1 and DAF-16/FOXO to stimulate directly the transcription of genes which influence the stress resistance [241]. Increased lifespan by decreased activity of the insulin/IGF1 pathway was also shown to be ire-1 or xbp-1 dependent. Xbp-1 is predicted to synergize with one or more transcription factors (e.g. DAF-16) in C.elegans [242]. Mice with deletion of (IRS)1 also show an increased lifespan [243]. So the insulin/IGF-1pathway has been shown to affect lifespan of worms, flies and mice, which strengthen the evidence for evolutionary conservation of mechanisms regulating lifespan. Recent studies in humans also suggest an association but the results are not yet conclusive; the strongest evidence to date is the FOXO3A gene. A variation in the gene of FOXO3A was shown to have a positive effect on the life expectancy of humans, and is found more often in centarians [244].

It is known that low levels of stressors like caloric restriction or thermal stress influence eukaryotic life span [245]. It has been reported that the beneficial effects of caloric restriction are mediated by sirtuins. Overexpression of sirtuins would extend lifespan [246]. In yeast, worms and flies, the lack of Sir2 (ortholog of mammalian SIRT1) inhibits the positive effect of caloric restriction on life span [247]. Likewise mice do not have an increased life span

by caloric restriction without SIRT1 [248, 249]. Recently it was found that some of the reported effects could be due to inconsistencies in experimental design. The lifespan increase as a result of dietary restriction in fly would be Sir2 independent, because two long lived strains as a result of sirtuin overexpression were not long lived when they were compared with another control strain. Furthermore, longevity and overexpression of sirtuins could be separated in C.elegans using genetic backcrossing. These results emphasize the importance of controlling genetic backgrounds. However, Sir2 overexpressing worms were more resistant to toxic protein aggregates, suggesting that sirtuins do have a positive effect on age-associated protein folding disease [250]. HSF1 is essential for longevity accomplished by dietary restriction in C.elegans [251]. Whereas HSF1 protein concentration increases with age, the amount of SIRT1 protein was shown to decrease with age [227, 252]. Together with a decrease in SIRT1 protein expression, HSF1 DNA binding and expression of heat shock proteins decrease [130].
HSF1 is important to protect cells from various stresses, to promote survival, and to extend life span. Yet, too much of HSF1 can also be deleterious. Higher resistance to stress could have a negative side effect by promoting development of cancer [253]. In addition, it is well known that human cancer cell lines show a greater dependence on HSF1 function than their normal counterparts [253].

7.2 The endoplasmic reticulum and UPR in aging

To date, only a few reports have implicated ER stress in the aging process. A study using a human diploid fibroblast cellular senescence culture model of aging showed an age-related decline in the expression of the ER chaperone calnexin, which is an essential component of ER-dependent protein folding. A decrease in calnexin could contribute to decreased cytoprotection [254]. Furthermore, three ER resident proteins, namely HSPA5, protein disulfide isomerase (PDI), and calreticulin show an age related increase in oxidative modifications. This implies a decline in protein folding, disulfide crosslinking and glycosylation during aging [255]. Another finding showed that cultured hepatocytes isolated from old rats were more sensitive to cell death induced by ER stress than their younger counterparts; this effect was c-Jun N-terminal protein kinase (JNK) dependent [256]. Activation of PERK and the inhibition of global protein synthesis by sleep deprivation is impaired in aged mice [257], which will result in more ER stress because new protein synthesis will raise the burden the ER. Sustained ER stress causes CHOP levels to increase and CHOP mediates apoptosis under ER stress conditions. Others have shown that young rat tissues displayed higher background of eIF2α phosphorylation plus a higher level of eIF2B to cope with the negative effect of eIF2α-P on translation. The decline in endogenous eIF2α phosphorylation in tissues from old rats is associated with increased GADD34 levels, and pro-apoptotic proteins such as CHOP. These results suggest that young

tissues possess an efficient ER stress adaptive mechanism that declines with aging [258]. The effect of proteasome inhibition on the UPR was studied in hippocampus from young and old rats. In hippocampus from young rats, IRE1α, ATF6 and PERK were active, while aged rats showed no activation of the IRE1α and ATF6 pathways. The PERK pathway was activated in young and old rats, which results in imbalanced pro-survival and pro-apoptotic protein concentrations [259].
The precise role of the ER and the UPR in the aging process is not fully identified yet. The ER plays vital roles in regulating overall protein homeostasis and cellular stress response pathways and the redox status is essential in regulating these functions, therefore the role of the ER in the normal aging process needs further investigation.

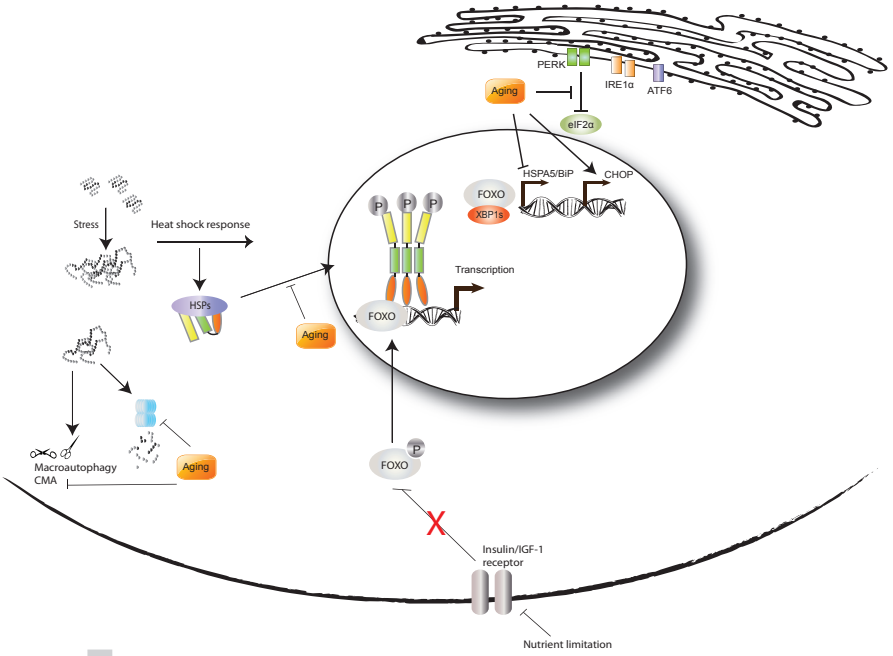


Figure 6 Proteotoxic stress responses and aging.
The DNA damage response is not shown for simplicity. UPR^{mt} has not been implicated in aging yet and a Golgi stress response pathway has also not been described. Only proteins with a role during aging are depicted (modified from [260]).

8 Scope of thesis

Cross-talk between the HSR and the UPR.
The HSR and the UPR are both activated by proteotoxic stress, although in different compartments, and share cellular resources. How these resources are allocated when both responses are active is not known. Insight in possible crosstalk will help understanding the consequences of failure of these systems in (age-related) disease. eIF2α phosphorylation is a mutual response to different types of stress. Cytosolic proteotoxic stress, ER stress, but also lack amino acids, all result in eIF2α phosphorylation. In chapter 2 we show that a heat shock results in selective synthesis of ATF4 just as ER stress does. A heat shock also induces IRE1-dependent XBP1 splicing. XBP1s inhibits the activity of the HSPB1 promoter as well as that of the Drosophila melanogaster Hsp70 promoter (Chapter 3). The DNAJB9 promoter (a known target of XBP1s) is also activated upon heat stress. Surprisingly, this activation is not via XBP1s, but requires an as yet unidentified transcription factor (Chapter 2). The responsiveness of a canonical heat shock promoter, HSPB1, to XBP1s and that of a canonical UPR promoter, DNAJB9, to heat stress suggest that there is crosstalk between the two stress systems.

Identification of critical nodes in the chaperoning network.
The only known way to upregulate the stress system is to cause cellular stress, which ultimately may be deleterious. To find ways to boost the defence and repair system without the deleterious effects, we need to know more about the system, its critical nodes and rate limiting steps. Depleting cells of cytoplasmic chaperones by overexpression of a dominant negative HSF1 (dnHSF1) mutant results in an inhibition of the glucocorticoid response. We show that the glucocorticoid response in chaperone depleted cells can be restored by DNAJA1 or B1 but not by HSP90AA1 or HSPA1A (Chapter 4). Overexpression of dnHSF1 also results in a lesser folding capacity in all cellular compartments, including the ER and peroxisomes. This system provides a good model to pinpoint critical nodes of the chaperoning network in each cellular compartment (Chapter 5).

Which genes are under control of HSF1 under non-stress conditions?
An unexpected target of dnHSF1 is phosphomevalonate kinase (PMVK), an enzyme in the cholesterol biosynthetic pathway. We show that the PMVK gene contains an HSE in the region encoding the 5' UTR. PMVK mRNA and protein levels were strongly downregulated not only when dnHSF1 (HSF379) was expressed, but also when an HSF1 mutant (HSF448), which has a weak dominant positive effect on traditional HSF1 responsive promoters, was expressed. PMVK transcript levels, although downregulated by dnHSF1 and HSF448, are not upregulated during proteotoxic stress. The activation of the PMVK promoter by sterol depletion was also not HSF1 de-

pendent. Whether the PMVK HSE plays a physiological role remains unclear (Chapter 6).

The microarray analysis of HEK293 cells overexpressing dnHSF1 resulted in a set of genes that are likely to be direct targets of HSF1. Comparison of our microarray results with the published data using HSF-/- cells (siRNA or knock-out) showed far fewer transcriptome changes in the dnHSF cells, suggesting that HSF1 does participate in gene regulation even when not activated. We used another model system in which cells still express HSF1, but in an inactive form. To that end we have used the HSF1-K80Q mutant which is unable to bind DNA (Chapter 7). Using this model we determined which genes are under control of HSF1 under non-stress conditions. The aging cell differs from the HSF1-/- cells in that the ageing cell still contains HSF1, although inactive, and differs from the dnHSF1 cells in that HSF1 is no longer bound to its target promoters. The model system using HSF1-K80Q could be a closer mimic of the aging cell to further probe the change in stress response with age. However, we found almost no overlap between the genes of which the transcript level changed significantly in non-stressed HeLa cells upon siRNA HSF1 treatment or upon overexpression of HSF1- K80Q in HEK293 cells. Either the genes controlled by HSF1 in the non-stressed state are largely cell specific or the effect of deleting HSF1 from a cell is significantly different from blocking HSF1 activity by overexpression of a non-DNA binding HSF1 mutant (Chapter 7).

What are the consequences of loss of regulation by HSF1 for cellular robustness?

HSF1-K80Q and dnHSF1 inhibited recovery from heat shock and some stress induced transcripts remained high in cells allowed to recover from heat shock for 24 hrs. The activity of some transcriptions factors, which normally decays after heat shock, is maintained when HSF1 activity is blocked (Chapter 7). In the final chapter, the results described in this thesis are summarized and discussed.

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Chapter 2

An atypical unfolded protein response in heat shocked cells

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Abstract

The heat shock response (HSR) and the unfolded protein response (UPR) are both activated by proteotoxic stress, although in different compartments, and share cellular resources. How these resources are allocated when both responses are active is not known. Insight in possible crosstalk will help understanding the consequences of failure of these systems in (age-related) disease. In heat stressed HEK293 cells synthesis of the canonical UPR transcription factors XBP1s and ATF4 was detected as well as HSF1 independent activation of the promoters of the ER resident chaperones HSPA5 (BiP) and DNAJB9 (ERdj4). However, the heat stress activation of the DNAJB9 promoter, a XBP1s target, was not blocked in cells expressing a dominant negative IRE1α mutant, and thus did not require XBP1s. Furthermore, the DNA element required for heat stress activation of the DNAJB9 promoter is distinct from the ATF4 and ATF6 target elements; even though inhibition of eIF2α phosphorylation resulted in a decreased activation of the DNAJB9 promoter upon heat stress, suggesting a role for an eIF2α phosphorylation dependent product. The initial step in the UPR, synthesis of transcription factors, is activated by heat stress but the second step, transcriptional transactivation by these factors, is blocked and these pathways of the UPR are thus not productive. Expression of canonical ER chaperones is part of the response of heat stressed cells but another set of transcription factors has been recruited to regulate expression of these ER chaperones.

Introduction

All cells contain an extensive network of chaperones to maintain proteostasis. When proteostasis is disturbed, additional chaperones are synthesized to restore protein folding or to increase removal of irreversibly unfolded proteins by targeting these for degradation. For reviews see [1-4]. Eukaryotic cells have two evolutionarily highly conserved systems to combat proteotoxic stress: the heat shock (HS) system and the unfolded protein response (UPR). The HS system is the major response to stress conditions in the cytosol [5], while cells respond to the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum by activating the UPR.

The UPR induces a transient attenuation of protein synthesis and a transcriptional activation of genes to expand the protein-folding capacity of the ER. These responses are mediated by three ER-localized transmembrane proteins: inositol requiring 1α (IRE1 α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [6-9]. Under non-stressed conditions, these proteins are sequestered by the chaperone HSPA5 (BiP). Unfolded proteins in the ER compete for HSPA5 and IRE1α, PERK and ATF6 are released [10]. Activation of IRE1α results in the removal of a 26-nucleotide intron from XBP1 mRNA allowing the synthesis of the transcription factor XBP1 [11,12]. Activation of PERK, an eIF2α kinase, leads to phosphorylation of eIF2α and thus to an overall inhibition of the initiation of protein synthesis [13]. Paradoxically, it also results in the preferential translation of some downstream ORFs, known as stress induced leaky scanning [14]. Stress induced leaky scanning is essential for the translation of the ATF4 ORF [15,16]. In addition to ATF4 mRNA, GADD34 [17] and ATF5 ORFs [18,19] are also subject to translational upregulation in response to eIF2α phosphorylation. GADD34 is a regulatory subunit of protein phosphatase I and mediates eIF2α -P dephosphorylation. The gene for GADD34 is also one of the targets of ATF4. GADD34 is thus part of a feedback loop [20,21]. ATF4, together with XBP1s and ATF6, directs the transcriptional response of the UPR. The heat shock response (HSR) shows some parallels with the UPR. The HSR is mediated by a single transcription factor, heat shock factor 1 (HSF1) [22]. Like the mediators of the UPR, HSF1 is sequestered by chaperones. In unstressed cells HSF1 is in the cytoplasm in a complex containing the chaperone Hsp90. Unfolding proteins compete for Hsp90 and upon its release from the Hsp90 complex HSF1 is activated [23-26]. HSF1 enhances the transcription of the so-called heat shock genes, genes that encode cytoplasmic chaperones such as HSPA1A (Hsp70), DNAJB1 (Hsp40) and HSPB1 (Hsp27) [27]. Like the UPR, a heat shock also results in activation of an eIF2α kinase, in this case both PKR and HRI [28,29]. In addition initiation of translation is inhibited through inhibition of the cap-binding complex [30-32].

It is likely that there is cross-talk between the HSR and the UPR. These

two responses share a resource, the proteasome, which degrades both the irreversibly folded cytoplasmic and ER proteins – the latter via the (ER)-associated degradation (ERAD) pathway [33] - and they share the eIF2α kinase regulatory pathway. The HSR and the UPR also compete for resources in the, not unlikely, case that a stressor causes protein unfolding in both cellular compartments. Indeed, a heat stress has been shown to transiently induce XBP1 splicing [34] and to lead to an increase in HSPA5 and DNAJB9 (ERdj4) mRNA levels [35], both typical UPR responses. We show here that heat stress induces an UPR like response, but that this response is not productive. The activation of the HSPA5 and DNAJB9 promoters is a late response of cells recovering from heat stress and the DNAJB9 promoter is not activated through the usual UPR induced transcription factors.

Materials and Methods

Cell culture

Flp-In T-REx-HEK293 cells (Invitrogen) were manipulated according to the manufacturer's instructions to generate the stable cell lines HEK-dnIRE1α, HEK-dnHSF1 and HEK-cDNA5, which carry a single copy of the tetracycline-inducible plasmids pcDNA5- dnIRE1α, pcDNA5-dnHSF1, and pcDNA5-FRT/TO, respectively. The cells were cultured at 37°C in the presence of humidified 5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments.

Plasmid Construction

The C-terminal truncation mutant of dnHSF1 containing codons 1-379 from HSF1 was previously described [41]. The truncated dnIRE1α lacks the sequence of kinase and ribonuclease domains [55]. pGEM-T-hIRE1αDelC-29 was made by PCR amplifying the 1.75-kb IRE1α cDNA from HEK293 cDNA using the IRE1up and IRE1-delClow primers and cloning the PCR fragment into the pGEM-T vector. pcDNA5-FRT-TO-IRE1αDelC was made by cloning the 1.75-kb HindIII-XhoI fragment of pGEM-T-hIRE1αDelC-29 into the HindIII and XhoI sites of pcDNA5/FRT/TO. pcDNA5-dpHSF1 was made by digesting pcDNA5/FRT/TO-dnHSF1 with BamHI(bl) and XhoI(bl) resulting in HSF1 AA1-201, then the SmaI-BglII(bl) fragment from pOTB-HSF1 (C-terminal fragment of HSF1 AA316- 529) was inserted into HSF1 1-201, generating pcDNA5/FRT/TO-HSF1Δ202-315 (dpHSF1).

The reporter constructs were made in the pGL3 basic vector (Promega). The *Drosophila melanogaster* Hsp70 (Hsp70Ab), HSP70A1A (-313, +196), HSPB1 (-685, +36) and HSPA5 (-2742, +202) promoter constructs were described previously [41,32]. The HSPA1B (-573, +13), and the DNAJB9 (-375, +153) promoter clones were constructed by PCR on DNA isolated from

human lymphocytes cells using the primers listed in table 1. PCR fragments were cloned into pGEM-T-Easy vector (Promega) and, after sequencing; the promoter sequence was cloned into the pGL3 basic vector. DNAJB9 promoter deletion constructs were generated by digesting pGL3- DNAJB9 (-376, +153) with SacI and XhoI (-254, +153) or PmlI (-109, +153). Blunt ends were generated and ligated.

The XBP1s expression construct was generated by PCR on cDNA manufactured from total RNA isolated from Flp-In T-REx 293 cells which were exposed to tunicamycin stress. The PCR product was cloned into the pcDNA5/FRT/TO HindIII-XhoI sites.

pcDNA5/FRT/TO-ATF4-ORF was made by amplifying the 1200-bp ATF4 ORF fragment from pcDNA5/FRT/TO-ATF4cDNA. The PCR fragment was treated with HindIII/EcoRI(bl) and cloned into the HindII and BamH1(bl) sites sites of pcDNA5/FRT/TO. pcDNA3.1-ATF6α (1-373) was kindly provided by Prof. Dr. Kazutoshi Mori [56].

pCMV-ATF4cDNA-T2A-luciferase was made by cloning the 1333 fragment of the ATF4cDNA PCR product (via T-vector) into the HindIII and EcoRI sites of pCMC-T2A-Luc [57]. The constitutively active GADD34 construct has been previously described [58]. pGL3-promoter-UPRE and pGL3-promoter-ERSE were made by annealing the corresponding primers (see Table 1) and cloning the double-stranded oligo into the NheI and BglII sites of pGL3-promoter.

Reporter assays

At 24 h before transfection, 0.4x105 HEK-cDNA5, HEK-dnIRE1α or HEK-dnHSF1 cells were plated per well in a 24-well plate. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. 200 ng plasmid was transfected, 20 ng βactin-β-galactosidase was used as a transfection efficiency control. At 24 hours after transfection doxycyclin was added. At 48 hours after transfection cells were harvested or exposed to a stressor. Cells were harvested and lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 20 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus; Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 20 µl cell lysate was mixed with 50 µl luciferin solution (Promega) and luminescence was measured with the Lumat luminometer. All reporter gene assays were performed in triplicate. Relative activities of luciferase reporter genes were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies.

Total RNA isolation & RT-PCR

RNA isolation, DNase treatment of the RNA and the reverse transcriptase reaction were performed as described [32]. Cells were harvested after the treatments and at the times indicated, washed twice with ice-cold PBS and after washing the cells, RNA was isolated with TRIzol (Invitrogen) according to manufacturer's recommendation. For QPCR analysis, cDNA was 10-fold diluted. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in table 1. To amplify XBP1 cDNA, PCR was for 32 cycles (95 °C for 30s; 58 °C for 30s; and 72 °C for 2 min or 4 min in the final cycle) using XBP1 PCR up and low oligo's with Taq DNA polymerase. 398 and 424 bp fragments representing spliced (XBP1s) and unspliced (XBP1u) XBP1, plus a hybrid (XBP1h) migrating as a fragment of approximately 450 bp, were documented after staining 3% agarose gels with ethidium bromide. XBP1h represents a mixture of two hybrid structures. Each structure contains one strand from XBP1s and one strand from XBP1u and is formed in the final annealing PCR step [59]. XBP1h has also been observed by others [60,61].

Western blotting

Immunoblot analysis was performed with cell lysates from HEK-cDNA5 and HEK-dnHSF1 cells as described previously [41]. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1: 15,000 dilution, HSPA1A antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal HSPA5 antibody, kindly donated by Prof. Dr. Ineke Braakman, was used at a dilution of 1:1000, polyclonal DNAJB1 antibody (anti-Hsp40; SPA-400; Stressgen) at a 1:10,000 dilution, HSPB1 antibody, obtained from Dr. A. Zantema, at a dilution of 1:400, polyclonal XBP1 antibody [(M-186): sc-7160; Santa Cruz Biotechnology], at a dilution of 1:200, polyclonal ATF4 antibody [(C-20): sc-200; Santa Cruz Biotechnology], at a dilution of 1:1000, monoclonal eIF2α antibody was at a 1:500 dilution, polyclonal phosphorylated eIF2α antibody (E2152; Sigma) was used at a 1:1,000 dilution, monoclonal γ-tubulin antibody (GTU-88; Abcam) at 1:1000 dilution and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye® 800CW conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32210, respectively; LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

Table 1 Oligonucleotides that were used.

Oligo name	Oligo sequence (5' → 3')	Oligo name	Oligo sequence (5' → 3')
IRE1 up	agctaagcttaccatgccggcccggcggct	EDEM QPCR up	aaagattccaccgtccaagtc
IRE1delC low	agctctcgagtcatccatggcccaggacatccttg	EDEM QPCR low	gtatcattgctccggaggtt
HSPA1B promoter up	agctctcgagaactatattgcattatctctttcct	HSPA5 QPCR up	ggccgcacgtggaatgac
HSPA1B promoter low	agctagatctggccgttttccggac	HSPA5 QPCR low	tccaatatcaacttgaatgtatgg
DNAJB9 promoter up	aacgcgtcttacaacaaatcgctgcgcaatag	HSPC4 (GRP94) QPCR up	ctgggactgggaacttatgaatg
DNAJB9 promoter low	accatggtggcgctggcgaccctgacgatc	HSPC4 (GRP94) QPCR low	tccatattcgtcaaacagacca
XBP1s expression up	agctaagcttaccatggtggtggtggcagccgc	CHOP QPCR up	accaagggagaaccaggaaacg
XBP1s expression low	agctctcgagttagacactaatcagctggggaa	CHOP QPCR low	tcaccattcggtcaatcagagc
XBP1 PCR up	ctggaacagcaagtggtaga	GADD34 QPCR up	cctctacttctgccttgtctccag
XBP1 PCR low	tctacccagaaggacccagt	GADD34 QPCR low	ttttcctccttctcctcggacg
hATF4 up	agctaagctttttctactttgcccgcccacag	DNAJB9 QPCR up	tggtggttccagtagacaaagg
hATF4 low	agctgaattcggggaccctttttcttcccct	DNAJB9 QPCR low	cttcgttgagtgacagtcctgc
pcDNA5-ATF4ORF up	agctaagcttctcacggcattcagcagc	DNAJB1 QPCR up	ttccccagacatcaagaacc
pcDNA5-ATF4ORF low	agctgaattccaccacactggactag	DNAJB1 QPCR low	accctctcatggtccacaac
ERSE up	ctagccgaccaatgatggtcgaccacgcgtgg	HSPA1A QPCR up	ccgagaaggacgagtttgag
ERSE low	gatcccacgcgtggtcgaccatcattggtcgg	HSPA1A QPCR low	acaaaaacagcaatcttggaaagg
UPRE RE up	ctagcacaggtgctgacgtggcattca	GAPDH up	ttccccatggtgtctgagc
UPRE RE low	gatctgaatgccacgtcagcacctgtg	GAPDH low	atcttcttttgcgtcgccag

Results

Activation of the eIF2α-phosphorylation dependent regulatory pathway during a heat shock.

During both ER stress and heat stress eIF2α is phosphorylated (Fig. 1A), where heat stress induced phosphorylation of eIF2α is most likely mediated by PKR and HRI, while ER stress activates PERK. When eIF2α is phosphorylated. ATF4 mRNA is selectively translated and a similar increase in ATF4 levels in cells recovering from heat shock and in DTT or tunicamycin treated cells was seen (Fig. 1B; the multiple ATF4 bands most likely represent phosphorylated forms [36]). eIF2α phosphorylation decayed rapidly in cells recovering from heat shock (Fig. 1A) and to show that the increase in ATF4 is indeed due to eIF2α-P dependent de novo translation, we designed reporter constructs in which translation of the luciferase code is dependent upon translation of the preceding ATF4 ORF by linking that ORF to the luciferase code with the T2A viral frameshift region. Upon translation of the T2A sequence, the ribosome frameshifts, releasing the protein, but continues translation of the downstream ORF [37,38], in our constructs the luciferase coding region (see fig. 1C). As expected, placing the ATF4 cDNA sequence, including the upstream ORFs, before the T2A-luciferase ORF was strongly inhibitory: the luciferase yield from the pCMV-ATF4 constructs was in unstressed cells about 5% of that obtained from the control, pCMV-T2A-luc (data not shown). Dephosphorylating the little eIF2α-P present in unstressed cells by expressing the C-terminal domain of GADD34 (C-term GADD34), a constitutively active mutant of the regulatory subunit of eIF2α dephosphorylase, caused a further decrease of the luciferase yield (the efficacy of exogenous expression of the C-term GADD34 in dephosphorylation of eIF2α-P

is shown in fig. 1D). Heat stressing the cells resulted in a sharp increase in luciferase yield from the ATF4 constructs, an increase that is completely prevented by exogenous expression of C-term GADD34 (Fig. 1E). These data show that the increase in ATF4 levels in heat stressed cells is due to eIF2α-P dependent translation initiation just as it is in ER stressed cells.

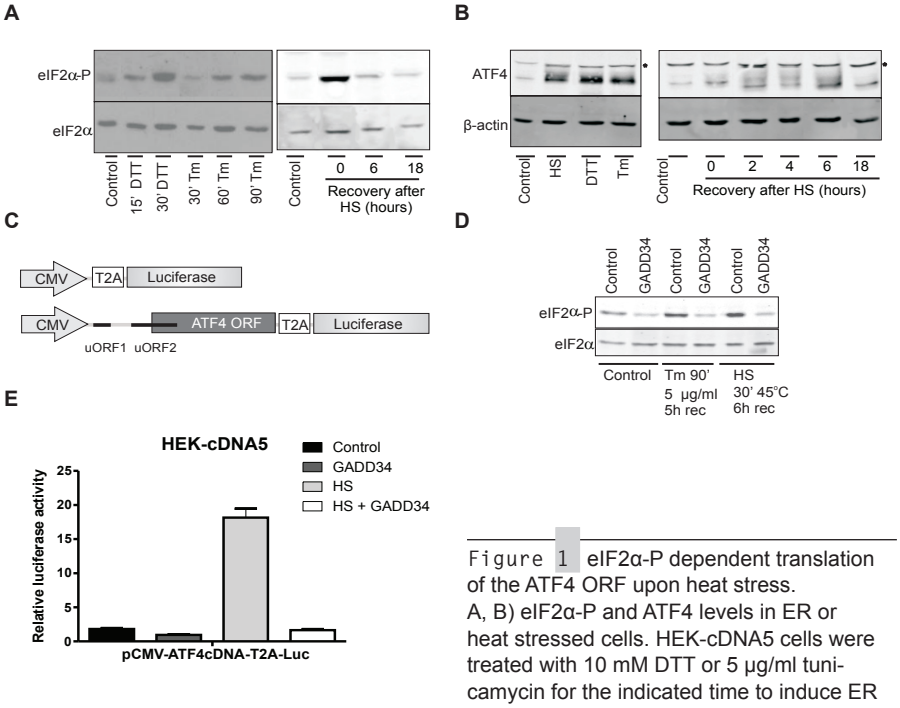


Figure 1 eIF2α-P dependent translation of the ATF4 ORF upon heat stress.
A, B) eIF2α-P and ATF4 levels in ER or heat stressed cells. HEK-cDNA5 cells were treated with 10 mM DTT or 5 µg/ml tunicamycin for the indicated time to induce ER stress. Alternatively cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of phosphorylated eIF2α (eIF2α-P) were determined by western blotting with eIF2α as a loading control (panel A) or levels of ATF4 were determined by western blotting with β-actin as a loading control (panel B). The asterisk indicates a non specific band. C) Schematic representation of the luciferase reporter constructs containing the ATF4 ORF. Translation of the luciferase code is dependent upon translation of the preceding ATF4 open reading frame. D) Expression of C-term GADD34 decreases the level of eIF2α-P. HEK-cDNA5 cells were transfected with GADD34 or with an empty vector. Cell lysates of unstressed HEK-cDNA5 cells or HEK-cDNA5 cells exposed to tunicamycin or HS were subjected to SDS-PAGE and levels of phosphorylated eIF2α (eIF2α-P) were determined by western blotting. eIF2α was used as a loading control. E) Translation of the ATF4 ORF in heat shocked cells is eIF2α-P dependent. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-β-gal reporter, and the expression construct for C-term GADD34 or an empty vector. At 48 h after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for 7 hours and harvested. Harvested cells were assayed for reporter gene activities. The results are the average of three independent transfections (standard deviations are indicated by error bars).

Activation of the ATF6 dependent regulatory pathway during a heat shock
The data presented above show that the ATF4 branch of the UPR is also activated in heat stressed cells. To determine whether a heat stress also activates the ATF6 arm of the UPR we used two reporter genes. One is driven by the UPRE [39]; the other by the ERSE [40]. Both DNA elements are targeted by ATF6 as evidenced by the increased activity upon exogenous expression of the soluble form of ATF6 (ATF6 1-373), but not uniquely, as these elements are also targets of XBP1s (Fig. 2A). To exclude an effect of XBP1s we inactivated this branch of the UPR by expression of a dominant negative mutant of IRE1α. Fig. 2B shows that expression of dnIRE1α inhibited tunicamycin induced XBP1 splicing. The activity of the UPRE reporter increased more than six fold after tunicamycin induced ER stress, while heat shock caused only a twofold induction. DnIRE1α expression strongly inhibited the ER stress induced activity, while the mild activation after heat shock was still observed (Fig. 2C left, black bars). The ERSE reporter was less responsive to stress and an increase of only twofold was seen after heat shock or tunicamycin treatment. In presence of dnIRE1α, tunicamycin no longer induced the reporter, while the heat shock induced activity remained. (Fig. 2C right, white bars) These results suggest that the increased activity of both the UPRE and the ERSE driven reporter genes in tunicamycin treated cells, but not in heat shocked cells, is dependent upon XBP1s. Only the UPRE driven reporter gene shows a slight activation in tunicamycin treated dnIREα cells, potentially due to ATF6. If so, ATF6 could also be responsible for the XBP1s independent activity seen in heat shocked cells. The activity of the ERSE driven reporter gene is completely dependent upon XBP1 in tunicamycin cells, no sign of a possible ATF6 contribution is seen. Hence we cannot conclude that the heat shock induced activity of this reporter is due to ATF6; it may well be due to other transcription factors.

Heat shock induces XBP1 mRNA splicing.
The XBP1 branch of the UPR has been reported to be activated in heat shocked cells [34], and we therefore expected to see an inhibition of the UPRE driven reporter gene in heat shocked dnIRE1α cells. However, we saw little effect (see Fig. 2C) and thus looked to see if XBP1 mRNA is spliced in heat shocked cells under our experimental conditions. Directly after heat shock or after 3 hrs of recovery, XBP1 mRNA was indeed completely spliced (Fig. 3A). After about 8 hrs unspliced XBP1 mRNA was again detected (Fig. 3B). Figure 3A shows that heat-induced XBP1 splicing was strongly inhibited by the expression of dnIRE1α, indicating that heat shock induced XBP1 splicing is, as expected, IRE1α dependent. Recovery from XBP1 splicing is dependent on a healthy heat shock system. In heat stressed MEF HSF1 -/- cells XBP1 splicing was prolonged [34]. In agreement, we found that expression of a dominant negative HSF1 mutant [41] also delays the reappearance of unspliced XBP1 after heat stress (Fig. 3B). XBP1 splicing in cells recovering from heat stress is transient and XBP1

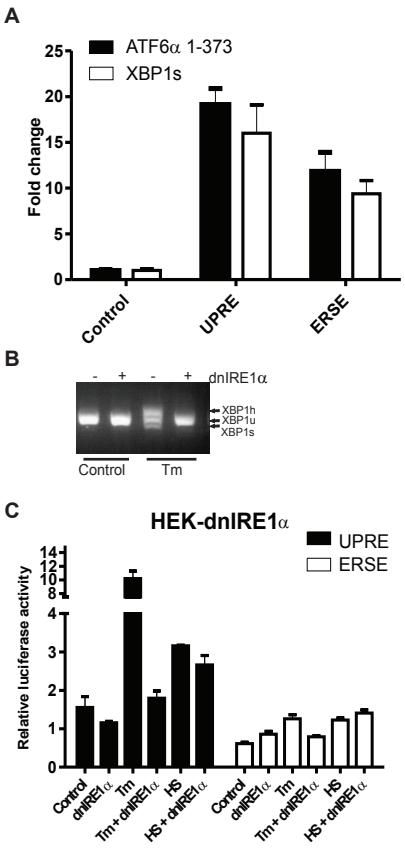


Figure 2 UPRE and ERSE directed reporter gene activity.
A) Effect of exogenous expression of ATF6 or spliced XBP1 on the activity of the UPRE-luciferase and ERSE-luciferase reporter constructs. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-β-gal reporter, and pcDNA3.1-ATF6α (1-373), pcDNA5-XBP1s or an empty vector pcDNA5/FRT/TO. At 48 hours after transfection, cells were harvested and assayed for reporter gene activities. B) dnIRE1α blocks XBP1 splicing. HEK-dnIRE1α cells were cultured in the absence or presence of doxycycline. Cells were treated with 10 μg/ml tunicamycin for 90' to induce XBP1 splicing. Total RNA samples were analyzed by RT-PCR. PCR products represent unspliced (XBP1u), spliced (XBP1s) and a hybrid of spliced and unspliced XBP1 PCR products (XBP1h; see also Materials and methods). C) HEK-cDNA5 and HEK-dnIRE1α cells were transfected with a mixture (9:1) of the indicated luciferase reporter and a βactin-βgal reporter. At 24 hours after transfection dnIRE1α expression was induced by adding doxycycline. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for 18 hours and harvested. To induce ER stress, cells were exposed to 2 μg/ml tunicamycin for 24 hours. Harvested cells were assayed for reporter gene activities.

protein can no longer be detected in cells that have recovered for 18 hrs from a heat shock, although it is present 6 hrs after heat shock (Fig. 3C).

The DNAJB9 promoter becomes activated upon heat stress, independent of XBP1s.

The data presented above show that the UPR is induced by heat shocking cells but leave some doubt as to whether the typical UPR transcriptional response is also seen: an UPRE driven reporter gene was only inhibited by dnIRE1α in tunicamycin treated cells, not in heat shocked cells (Fig. 2C). We thus tested the heat shock inducibility of two canonical UPR promoters, the human DNAJB9 (ERdj4) promoter and HSPA5 (BiP) promoter. The DNAJB9 promoter is a target of XBP1s [42,43] and ATF4 [44]; the HSPA5 promoter is activated by ATF6 via an ERSE [45] and also by ATF4 [46]. The ATF4, ATF6 and XBP1s sensitivity of our HSPA5 and DNAJB9 promoter constructs was tested by assaying their response to exogenous expression of either ATF4, ATF6 or XBP1s. The HSPA5 promoter was most sensitive to exogenous ATF6 expression (Fig. 4A, white bars) while the DNAJB9 promoter

responded best to exogenous XBP1s expression (Fig. 4A, light gray bars; see Fig. S1 for expression levels of exogenous XBP1s and ATF4 protein). In cells recovering from a heat shock for 6 hrs, luciferase constructs driven by either the DNAJB9 or the HSPA5 promoter were not active, while human HSPA1A (Hsp70) or HSPA1B promoter driven luciferase genes were fully active. However, a HSPB1 promoter driven luciferase gene was also inactive, even though the HSPB1 promoter is a canonical heat shock promoter (Fig. 4B). When the activity of the same constructs is assayed in cells that were allowed to recover from a heat shock overnight (18 hrs), the activity of the HSPA1 promoter driven constructs was already decaying (relative to pGL3 control), but that of the HSPB1, DNAJB9 and HSPA5 promoter constructs was higher. Apparently these three promoters are delayed responders to a heat shock. The delayed activity of at least the HSPB1 and HSPA5 promoter constructs reflects that of the endogenous genes: the protein products were only detectable in cells after overnight recovery from the heat shock (Fig. 4C). As expected, the activity of the HSPA5 and DNAJB9 promoters increased upon tunicamycin treatment of the cells, while that of the HSPA1A promoter did not (Fig. 4D).

The increased activity of the HSPA5 and DNAJB9 promoters in heat shocked cells raised the question whether other ER stress responsive genes are also activated. We thus looked at the changes in transcript levels of six ER stress responsive genes (HSPA5, DNAJB9, GRP94, EDEM, CHOP and GADD34) in heat shocked or tunicamycin treated cells and compared this with the changes in HSPA1A and DNAJB1 mRNA levels (Fig. 5). The

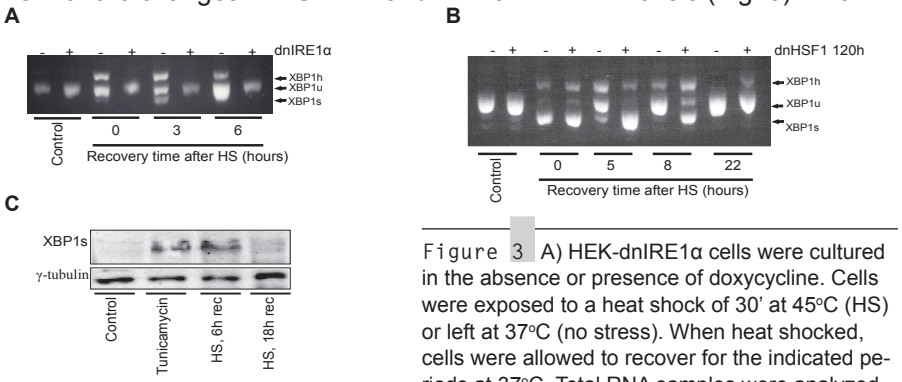


Figure 3 A) HEK-dnIRE1α cells were cultured in the absence or presence of doxycycline. Cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (no stress). When heat shocked, cells were allowed to recover for the indicated periods at 37°C. Total RNA samples were analyzed by RT-PCR. B) HEK-dnHSF1 cells were cultured in the absence or presence of doxycycline for the indicated time. Cells were exposed to a heat shock (30', 45°C), harvested at the indicated time point after heat shock, and subjected RT-PCR analysis to investigate the effect of HEK-dnHSF1 on XBP1 splicing after heat stress. C) XBP1s levels in heat stressed cells. HEK-cDNA5 cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for the indicated time before harvesting. To induce ER stress, cells were treated with 5 µg/ml tunicamycin for 90 minutes. After 5 hours recovery cells were harvested. Cell lysates were subjected to SDS-PAGE and levels of XBP1s were determined by western blotting with γ-tubulin as a loading control.

Figure 4 Stress response of various promoters. A) ATF4, ATF6 and XBP1s activate the HSPA5 and DNAJB9 promoters. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-β-gal reporter, and pcDNA5-XBP1s, pcDNA5-ATF4ORF, pcDNA3.1-ATF6α (1-373) or the empty vector pcDNA5/FRT/TO. At 24 hours after transfection doxycyclin was added to induce expression of XBP1s or ATF4. At 48 hours after transfection, cells were harvested and assayed for reporter gene activities. For the levels of exogenously expressed XBP1 and ATF4, see Fig. S1. B) Heat shock response of various promoters. HEK-cDNA5 cells were transfected with the indicated promoter reporter construct and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 h after transfection, cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for 6 hours or 18 hours, and assayed for reporter gene activities. The luciferase activity of the promoter constructs is relative to the activity of pGL3 control. C) HEK-cDNA5 cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of HSPA1A, HSPA5 and HSPB1 were determined by western blotting. D) ER stress activation of various promoters. HEK-cDNA5 cells were transfected with the indicated promoter reporter construct and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 h after transfection, cells were exposed to 2 µg/ml tunicamycin for 18 hours, and assayed for reporter gene activities. The luciferase activity of the promoter constructs is relative to the activity of pGL3 control.

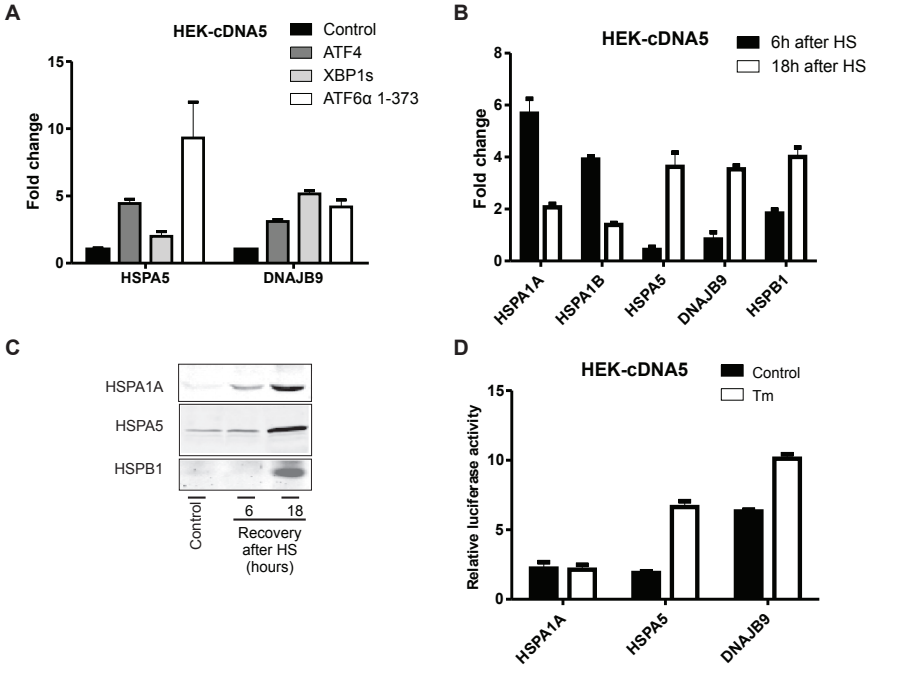
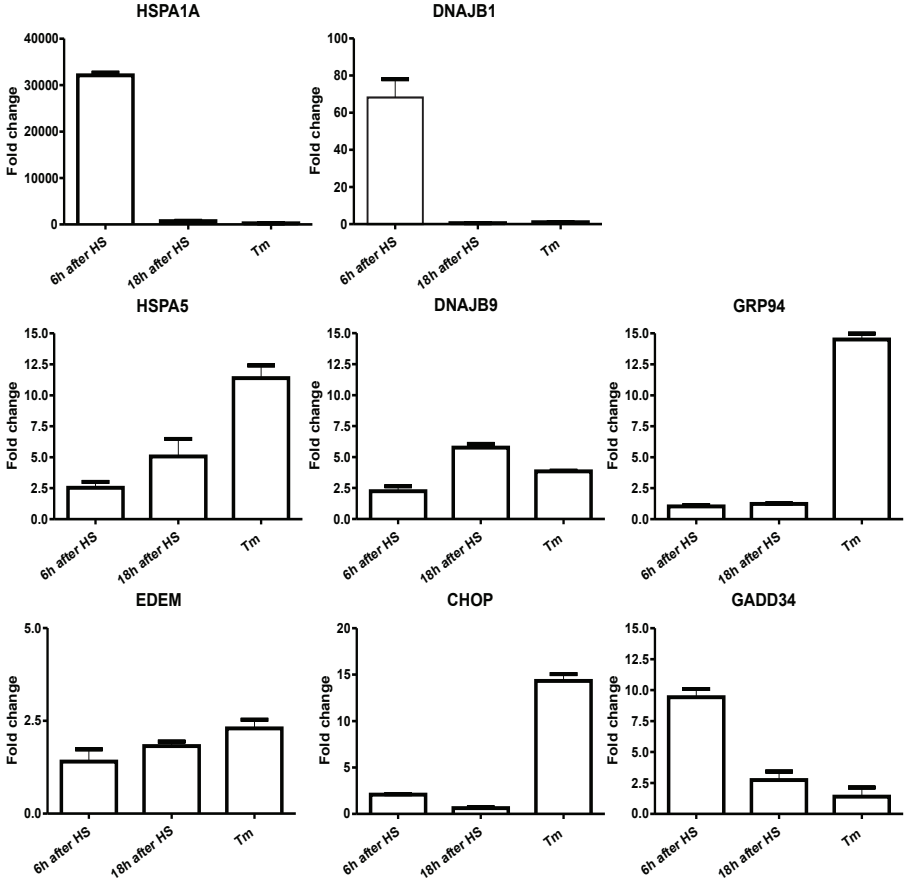


Figure 5 Relative changes in transcript levels of ER responsive genes in heat shocked and tunicamycin treated cells.
HEK-cDNA5 cells were treated with 2 µg/ml tunicamycin for 24 hours to induce ER stress. Alternatively cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels were measured by QPCR. Fold induction of mRNA levels is plotted relative to GAPDH mRNA levels.

transcript levels of the ER responsive genes all increased upon tunicamycin treatment and, with the exception of DNAJB9 and GADD34, were higher than in heat shocked cells. The HSPA1A and DNAJB1 mRNA levels did not increase in tunicamycin treated cells. A (modest) increase in the transcript level of all genes tested was seen in cells 6 hours after heat shock. Transcript levels were generally higher in cells 18 hrs after heat shock, with the exception of the two canonical ATF4 target genes (CHOP and GADD34) and the two canonical heat shock genes (HSPA1A and DNAJB1). These data show that these six ER responsive genes are active in heat shocked cells; generally, as also indicated by the activity of the promoter constructs, as a delayed response. The relative level of the transcripts in heat shocked cells is, however, different from that in tunicamycin treated cells (Fig. 5).
To determine whether the heat shock induced activity of the HSPA5 and DNAJB9 promoters (see Fig. 4B) is a result of the activity of the transcription factor HSF1, we tested promoter activity in HEK-dnHSF1 cells. As shown in figure 6B, the expression of dnHSF1 effectively blocks the heat shock induced activity of the DmHsp70Ab, the HSPA1A, the HSPA1B,



and the HSPB1 promoter constructs, but not that of the DNAJB9 promoter construct, while the HSPA5 promoter construct is only slightly inhibited (note that the levels of HSF1 and dnHSF1 remain constant in cells recovering from heat shock; Fig. S2). To confirm that the DNAJB9 and the HSPA5 promoter constructs do not respond to HSF1, we also tested the effect of a dominant positive HSF1 mutant. Expression of this mutant strongly upregulated the endogenous HSPA1A, DNAJB1 and HSPB1 protein levels, indicating an activated heat shock response (Fig. 6C). Expression of dpHSF1 also resulted in increased activity of the DmHsp70Ab and the HSPB1 promoter constructs

Figure 6 HSF1 dependency of various promoters. A) Expression of dnHSF1. HEK-dnHSF1 were cultured in the absence or presence of doxycycline to induce expression of dnHSF1. After harvesting the cells were subjected to SDS-PAGE and levels of endogeneous HSF1 and dnHSF1 were determined by western blotting with β-actin as a loading control. B) The effects of dnHSF1 on basal and heat shock induced activity of various promoters. Cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection doxycyclin was added to induce expression of dnHSF1. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for the indicated periods at 37°C. Harvested cells were assayed for reporter gene activities. C) Expression of dpHSF1 leads to increased HSP levels. HEK-cDNA5 cells were tranfected with pcDNA5-dpHSF1. At 24 hours after transfection cells were cultured in the absence or presence of doxycycline to induce expression of dpHSF1. At 48 hours after transfection cells were harvested and subjected to SDS-PAGE and levels of endogenous HSF1, dpHSF1, HSPA1A, DNAJB1 and HSPB1 were determined by western blotting. D) The effect of dpHSF1 on the activity of various promoters. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a βactin-βgal reporter, and pcDNA5-dpHSF1. At 24 hours after transfection doxycycline was added to induce expression of dpHSF1. At 48 hours after transfection, cells were harvested and assayed for reporter gene activities.

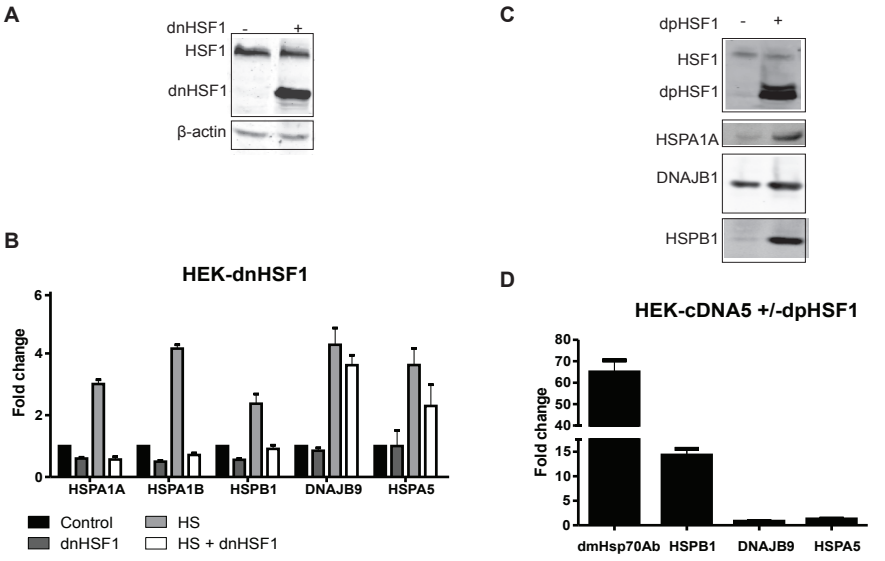
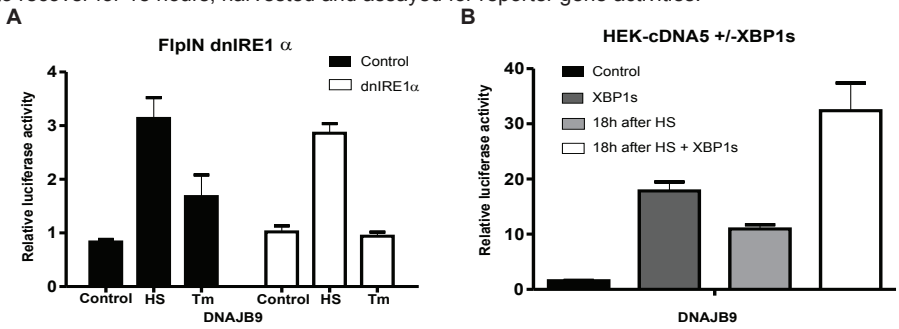


Figure 7 DNAJB9 promoter activity is not regulated by XBP1s in heat shocked cells. A) Activity of the DNAJB9 promoter in HEK-dnIRE1α cells. HEK-dnIRE1α cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection doxycyclin was added to induce expression of dnIRE1α. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for 18 hours and harvested. Alternatively, cells were exposed to 2 µg/ml Tunicamycin for 18 hours. Harvested cells were assayed for reporter gene activities. B) The DNAJB9 promoter can be activated by exogenous XBP1s. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-βgal reporter, and pcDNA5-XBP1s or the empty vector pcDNA5/FRT/TO. At 24 hours after transfection doxycyclin was added to induce expression of XBP1s. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for 18 hours, harvested and assayed for reporter gene activities.



in the absence of stress, yet no effect was seen for the DNAJB9 or HSPA5 promoter constructs (Fig. 6D). We conclude that the DNAJB9 and HSPA5 promoters are not a target of HSF1.

The activation of canonical UPR promoters in cells recovering from a heat shock does suggest that heat shocked cells mount the UPR response. We thus tested whether the DNAJB9 promoter construct is activated by a heat shock when the XBP1 branch of the UPR is blocked. As shown in figure 7A, in tunicamycin treated cells, activation of the DNAJB9 promoter construct is fully dependent upon XBP1s, as the response is absent in dnIRE1α cells. However, in heat shocked dnIRE1α cells, the DNAJB9 promoter construct is fully active. Hence endogenous XBP1s is not involved in the heat shock activation of the DNAJB9 promoter. XBP1s is not inactivated in heat shocked cells, as bypassing IRE1α by exogenous expression of XBP1s did increase the activity of the DNAJB9 promoter (Fig. 7B). One possibility is that the DNAJB9 promoter construct is activated by ATF4, which is also upregulated in heat shocked cells (Fig. 1). ATF4 synthesis requires eIF2α phosphorylation and we thus checked whether activation of the DNAJB9 construct was dependent upon eIF2α phosphorylation. As control we used the HSPA5 promoter construct, known to be induced by eIF2α phosphorylation [46]. As shown in figure 8, the activity of these two promoter constructs both in unstressed and in heat stressed cells was indeed blocked when eIF2α is dephosphorylated by exogenous expression of the C-terminal domain of GADD34, showing that the transcriptional activation is the consequence of

stress induced differential translation.
If the transcription factor involved in the heat shock activation of the DNAJB9 promoter is ATF4, then the ATF4 responsive element in that promoter should also be the element mediating the heat shock inducibility of the DNAJB9 promoter. The DNAJB9 promoter contains a CRE-like element around -140 and a CCAAT box around -65. ATF4 preferentially binds a CRE, but was also shown to bind CCAAT box although less efficiently [47]. Deleting the promoter to -109 resulted in loss of the heat shock inducibility but the truncated promoter could still be activated by ATF4 and XBP1s (Fig. 8). Hence the DNA region required for heat shock inducibility and that required for activation by ATF4 do not co-localize and we therefore conclude that the heat shock induction of the DNAJB9 promoter is not mediated by ATF4 (see Fig. 9). The heat insensitive DNAJB9 promoter construct was also still activated by exogenous expression of ATF6, also excluding ATF6 as the transcription factor responsible for DNAJB9 promoter activation after heat shock.

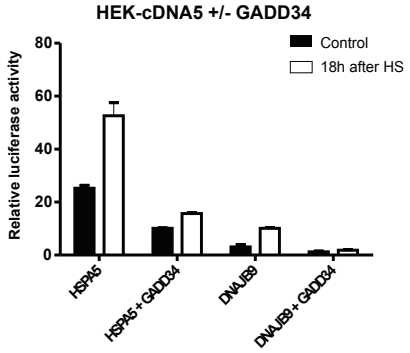


Figure 8 eIF2α-P dependent DNAJB9 and HSPA5 promoter activity. Activity of DNAJB9 and HSPA5 promoter in heat shocked cells is eIF2α-P dependent. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-β-gal reporter, and the expression construct for GADD34 or an empty vector. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for 18 hours and harvested. Harvested cells were assayed for reporter gene activities.

Discussion

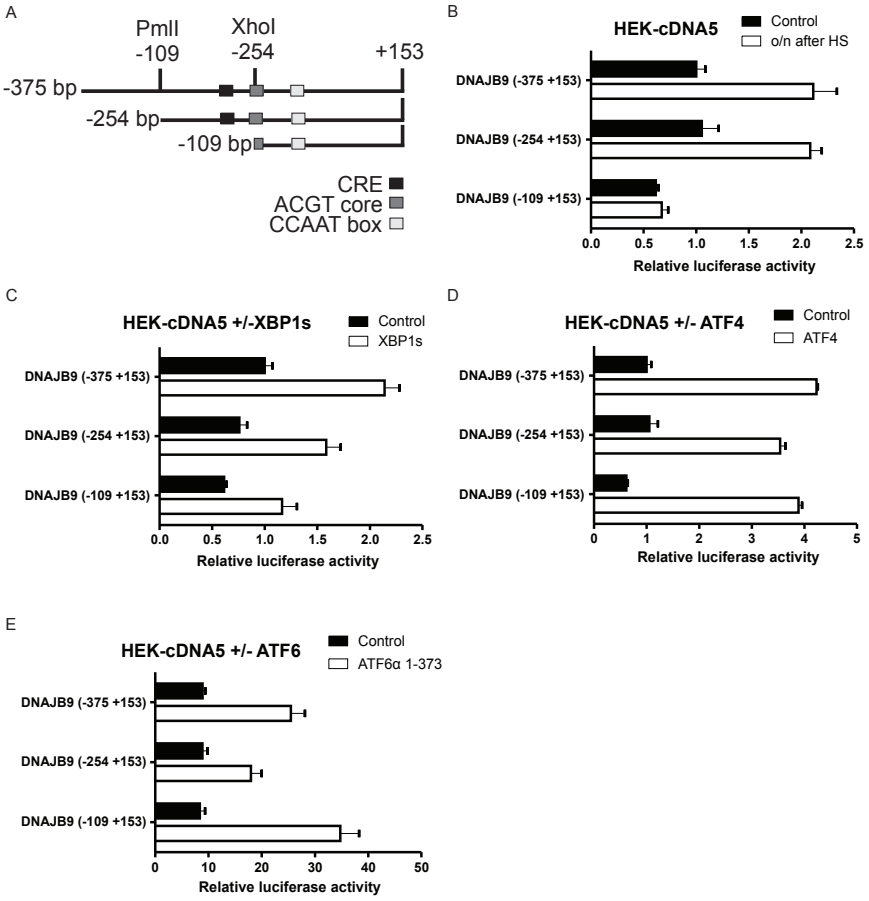
In agreement with a previous study [34], we found that a heat shock elicits the typical ER stress markers: phosphorylation of eIF2α and splicing of XBP1 mRNA. In addition, we show that ATF4 is synthesized, that XBP1s is made, that consensus ERSE and UPRE elements direct transcriptional activation in heat shocked cells and that canonical UPR promoters, HSPA5 and DNAJB9, are activated in heat shocked cells as also seen previously in microarray studies [35]. The response resembles a typical UPR, albeit with some differences in the relative mRNA levels. However, when we tried to identify the transcription factors involved in the transcriptional activation of UPR promoters in heat shocked cells, we found that those factors differ from the traditional ones responsible for the UPR (ATF4, XBP1 and ATF6). XBP1s plays a major role in the transcriptional activation directed by the ERSE or UPRE elements in tunicamycin treated cells, but is not involved in the transcriptional activation directed by these elements after a heat shock as the heat shock response is not blocked in dnIRE1α cells. Similarly, when the response of the DNAJB9 promoter is further dissected, then it is clear

that the activation of this promoter during heat shock is distinct from the activation during the UPR: deleting the promoter to -109 does not affect the activation by ATF4 or ATF6, has some effect on the activation by XBP1s but the heat shock induction is lost. Hence neither ATF4, ATF6 nor XBP1s plays a role in the heat shock activation of this promoter. Our experiments also exclude that the DNAJB9 promoter is a target of HSF1. As blocking eIF2α phosphorylation also blocks the heat shock response of the DNAJB9 (and of the HSPA5) promoter, the activity of the transcription factor responsible is somehow translationally controlled. We have tested ATF5 and CHOP (a target of ATF4) but neither activated either the DNAJB9 or the HSPA5 promoter (data not shown).

eIF2α phosphorylation is a common response to different stressors, not only cytoplasmic or ER proteotoxic stress, but also lack of amino acids [48]. ATF4 synthesis is thus also common to various types of stress and the pattern of transcriptional activation by ATF4 needs to be tailored to fit the type of stress. The targets of ATF4 are thus also determined by stress specific auxiliary factors [49]. Therefore, it is perhaps not surprising that ATF4 does not transactivate all its usual UPR targets during the heat shock response. XBP1s synthesis however is uniquely controlled by IRE1α dependent splicing and we expected XBP1s to be active irrespective of other stress responses. XBP1s transactivation activity itself is not blocked in heat shocked cells, as exogenous XBP1s does activate the DNAJB9 promoter construct in heat shocked cells (Fig. 7). Endogenous XBP1s activity in heat shocked cells is predicted to be transient. We find only a minor fraction of spliced XBP1 mRNA in cells recovering for 5 hrs from a heat shock and we do not detect XBP1s in cells that have recovered for 18 hrs. It is possible that the activity of endogenous XBP1s is masked by that of another transcription factor targeting the same DNAJB9 promoter. Alternatively, endogenous XBP1s may be inactivated by acetylation [50] or sumoylation [51] during the first few hours after heat shock. Exogenous XBP1s, which continues to be expressed, might escape from such inhibition at later times.

At least in yeast the UPR becomes activated during heat stress, in addition to the heat shock response [52,53]. Here, the heat shock response and the UPR cooperate and the heat shock response can rescue a defective UPR [54]. Our data suggest that after a heat shock mammalian cells first devote their resources to increasing the cytoplasmic chaperoning capacity - and only later switch to augmenting the ER chaperones. Accumulation of HSPA1A is detected within 6 hrs after heat shock, while HSPA5 levels increase later (Fig.4). At the time that the DNAJB9 and the HSPA5 promoters become active, the heat shocked induced UPR (as indicated by XBP1 splicing) has already decayed. Perhaps that is why another set of transcription factors needed to be recruited. This set of transcription factors could also be active in ER-stressed cells, and thus be part of the traditional UPR response, but have gone undetected because of the overlapping ATF4, ATF6 and XBP1 activity.

Figure 9 DNAJB9 promoter deletion constructs. A) Schematic representation of the DNAJB9 promoter region. B) Heat shock inducibility of DNAJB9 deletion constructs. HEK-pcDNA5 cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for 18 hours, harvested and assayed for reporter gene activities. C,D,E) Effect of exogenous XBP1s, ATF4 or ATF6 expression on the activity of promoter deletion constructs. HEK-cDNA5 cells were transfected with a mixture (4:1:5) of the indicated luciferase reporter, a β-actin-β-gal reporter, and an expression construct as indicated or the empty vector pcDNA5/FRT/TO. At 24 hours after transfection doxcycyclin was added to induce XBP1s C) or ATF4 expression D). At 48 hours after transfection, cells were harvested and assayed for reporter gene activities.



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Supplemental tables and figures

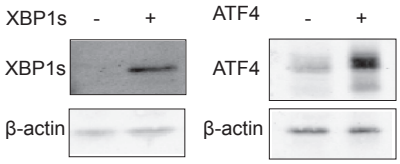

Figure S1
The levels of exogenously expressed XBP1s (left panel) or ATF4 (right panel) were determined by western blotting with β-actin as a loading control. See also legend to Fig. 4A.

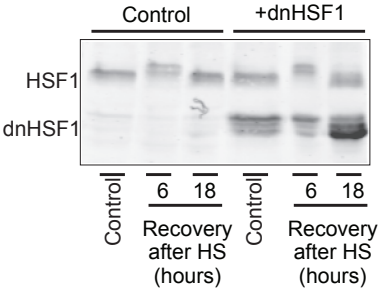

Figure S2
The levels of dnHSF1 and endogenous HSF1 in HEK-pcDNA5 and HEK-dnHSF1 were determined by western blotting. Cells were harvested after heat shock at the times indicated. Equal amounts of cellular protein were loaded.

Chapter 3

XBP1s modulates the expression of some HSF1 target genes

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Manuscript in preparation

Abstract

A heat shock also evokes the unfolded protein response and thus the production of spliced XBP1 protein (XBP1s). To address the question whether the activation of XBP1 during heat stress has an effect on typical heat shock genes, we tested the effect of overexpressing XBP1s on the activation of genes involved in the heat shock response. We show here that XBP1s inhibited the expression of HSPB1 in non stressed and stressed cells. XBP1s also inhibited the activity of HSPB1 and Drosophila melanogaster Hsp70 promoter constructs after heat stress, including constructs that only contained the HSE region. The BAG3 and SERPINH1 genes were found to be targets of both HSF1 and XBP1 in published ChIP-on-chip experiments. The heat stress induced transcriptional activation directed by either the BAG3 or the SERPINH1 heat shock element in promoter constructs was inhibited by XBP1s but at the mRNA levels XBP1s enhanced the heat shock induced increase in BAG3 or SERPINH1 transcripts. XBP1s did bind to the HSE containing promoter regions of BAG3 and SERPINH1 as assayed by ChIP. XBP1s binding decreased in heat shocked cells, suggesting that XBP1s might be inactivated by a heat shock. Together our data show that XBP1s modulates the expression of some HSF1 target genes; an effect that appears to be mediated by the HSE containing region.

Introduction

The heat shock (HS) response and unfolded protein response (UPR) are two fundamental, yet distinct, cellular responses that are evoked by proteotoxic stress. The HS response is activated by proteotoxic stress in the cytoplasm and is mainly regulated at the level of transcription by heat shock factor 1 (HSF1) [1]. Proteotoxic stress in the ER activates the UPR. In mammals the UPR consists of three arms, regulated by three ER transmembrane proteins: inositol requiring 1 (IRE1α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). The ER chaperone protein HSPA5 serves as a master UPR regulator and plays an essential role in repression of IRE1α, PERK and ATF6 by binding to these proteins in non stressed cells. Upon accumulation of unfolded proteins in the ER, the unfolded proteins bind and sequester HSPA5, thus freeing IRE1α, PERK and ATF6 to elicit their activation. Active IRE1α initiates a spliceosomal-independent mRNA splicing and removes a 26-nucleotide intron from unspliced mammalian XBP1 mRNA. This introduces a frameshift and leads to the production of active XBP1s, a basic leucine zipper type transcription factor [2]. The splicing of XBP1 mRNA is a central mechanism of the IRE1α pathway [3]. XBP1s is not only part of a stress response system; it has also other roles. In some organs and cells XBP1s is required for protein secretion [4-6] and XBP1s was shown to control growth and survival of hepatocytes [7]. There are conflicting reports about the role of XBP1s in maintaining cellular integrity. XBP1s has been implicated to influence cell survival positively [8-12]. However, others suggest a negative role for XBP1s in cell survival [8, 13, 14]. These conflicting results stress that the role of XBP1s is condition and cell type dependent. Therefore expression of XBP1s is tightly regulated. We have previously shown that heat stress does activate IRE1α and that splicing of XBP1 mRNA can be detected in heat stressed cells [15]. However, the XBP1s target gene DNAJB9 (ERdj4) was not activated by XBP1s – its activation in heat shocked cells is mediated by other factors. This finding made us wonder whether XBP1s does affect the heat shock response at all. We thus tested the effect of inhibiting XBP1 splicing or the effect of overexpressing XBP1s on the activation of genes involved in the heat shock response. We show here that XBP1s does affect the expression of at least some HSF1 target genes, an effect that appears to be mediated by the HSE, the DNA binding site of HSF1.

Materials and Methods

Cell culture

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-dnIRE1α, HEK-XBP1s and HEK-cDNA5 that carry a single copy of the tetracycline-inducible plasmids pcDNA5- dnIRE1α,

pcDNA5-XBP1s and pcDNA5-FRT/TO, respectively (see also [15, 16]). The cells were cultured at 37°C in the presence of humified 5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments.

Plasmid Construction

pcDNA5-dnIRE1α, has been described previously [15]. The XBP1s expression construct was generated by PCR on cDNA manufactured from total RNA isolated from Flp-In T-REx 293 cells which were exposed to tunicamycin stress. The PCR products were cloned into the pcDNA5/ FRT/TO HindIII-XhoI sites.

The reporter constructs were made in the pGL3 basic vector (Promega). The Drosophila melanogaster Hsp70 (Hsp70Ab), the HSPB1 (-685, +36) promoter construct and the DNAJB9 (-375, +153) promoter construct were described previously [15-17]. The HSPB1 (-1098, +36) promoter region was isolated by PCR on DNA isolated from human lymphocytes cells and inserted in the pGL3 basic vector. The HSPB1 (-210, +36) reporter construct was made by digesting the HSPB1 (1098, +36) promoter with KpnI-EcoRI (blunt). pGL3-promoter-Hsp70Ab HSE and pGL3-promoter-HSPB1 HSE were made by annealing the corresponding primers and cloning the double-stranded oligo into the BglII and NheI sites of pGL3-promoter. pGL3-promoter-SERPINH1 HSE, pGL3-promoter- BAG3 Δ HSE and pGL3-promoter-BAG3 HSE were made by annealing the corresponding primers and cloning the double-stranded oligo into the NheI and XhoI sites of pGL3-promoter.

Reporter assays

In general: at 24 h before transfection, 0.4x105 HEK-cDNA5 or HEK-dnIRE1α or HEK-XBP1s cells were plated per well in a 24-well plate. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. 200 ng plasmid was transfected of which 20 ng was β-actin-β-galactosidase as a transfection efficiency control. At 24 hours after transfection doxycycline was added unless indicated differently. At 48 hours after transfection cells were harvested or exposed to a stressor before harvesting. Cells were harvested and lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 20 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus; Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 20 µl cell lysate was mixed with 50 µl luciferin solution (Promega) and luminescence was measured with the Lumat luminometer. All reporter gene assays were per-

formed in triplicate.

Western blotting

Immunoblot analysis was performed with cell lysates from HEK-dnIRE1α, HEK-XBP1s and HEK-cDNA5 cells. Cell pellets were homogenized in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4x sample buffer (200 mM Tris–HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95oC for 5 min. Protein samples were separated in 12% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1: 15,000 dilution, HSPA1A antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal DNAJB1 antibody (anti-Hsp40; SPA-400; Stressgen) at a 1:10,000 dilution, monoclonal HSP90AA1 antibody (610418, BD Biosciences) at a 1:1,000 dilution, HSPB1 antibody, obtained from Dr. A. Zantema, at a dilution of 1:400, polyclonal HSPA5 antibody, kindly donated by Prof. Dr. Ineke Braakman, was used at a dilution of 1:1000, polyclonal XBP1 antibody (M-186): sc-7160; Santa Cruz Biotechnology), at a dilution of 1:200, polyclonal ST13 antibody (ab13490; Abcam) at a 1:1,000 dilution, polyclonal STIP1 antibody (ab65046; Abcam) a 1:1,000 dilution, polyclonal BAG3 antibody, kindly donated by Prof. Dr. Harrie Kampinga, was used at a dilution of 1:2000, monoclonal γ-tubulin antibody at 1:1000 dilution (GTU-88; Abcam) and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye® 800CW conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32210, respectively; LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

QPCR analysis

Cells were harvested after the treatments and at the times indicated, washed twice with ice-cold PBS and after washing the cells, RNA was isolated with TRIzol (Invitrogen) according to manufacturer's recommendation. 1 μg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 μM oligo(dT) were added to a total volume of 20 μl. Reverse transcription was performed for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with Power

SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in Table 1.

Chromatin Immunoprecipitation

HEK-XBP1s or HEK-cDNA5 cells were cultured for 24 h in the presence of doxycycline. Chromatin immunoprecipitation was performed as described in [18] except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES-KOH pH 7.6, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor complete). Sonicated chromatin was centrifuged for 5 min at 4°C and then incubated overnight in incubation buffer (final concentration; 12 mM HEPES-KOH pH 7.6, 90 mM NaCl, 0.6 mM EDTA pH 8.0, 0.09% SDS, 0.6% Triton X-100, 0.1% BSA) together with purified anti-HSF1 antibody (SPA-901; Stressgen) or XBP1 antibody ([M-186]: sc-7160; Santa Cruz Biotechnology) and protein A/G beads (Santa Cruz Biotechnology). Negative control without adding antibody was included. Beads were washed six times with different buffers at 4°C: twice with 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 150 mM NaCl, HEG (1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES-KOH pH 7.6), once with the same buffer but with 500 mM NaCl, once with 0.25 M LiCl, 0.5% NaDOC, 0.5% NP-40, HEG and twice with HEG. Precipitated chromatin was eluted with 400 µl of elution buffer (1% SDS, 0.1 M NaHCO3), incubated at 65°C for 4 h in the presence of 200 mM NaCl, phenol extracted and precipitated with 20 µg of glycogen at -20°C overnight. ChIP experiments were analyzed by qPCR. Efficiency of ChIP was calculated as percentage of input. The primers used are listed in Table 1.

Results

The effect of XBP1s on heat shock responsive genes.

To uncouple the synthesis of XBP1s from a proteotoxic stress response, we placed the XBP1s coding sequence under the control of a tetracycline inducible promoter in T-REx HEK293 cells, thereby making XBP1s expression independent of activation of IRE1α and ensuring continuous expression of XBP1s. Induction of expression of XBP1s led to the expected increase in XBP1 mRNA and protein as well as an increase in the transcript levels of the UPR target genes DNAJB9 and HSPA5. The HSPA5 protein levels increased accordingly (Fig. S1). Together these data show that XBP1s is active. We then tested whether turning on expression of XBP1s in otherwise non-stressed cells had an effect on HSF1 target genes. To our surprise, expression of XBP1s resulted in a rapid loss of the HSPB1 mRNA and protein (Fig. 1A, B). The protein levels of other HSF1 targets, such as DNAJB1, did not

Table 1 Oligonucleotides that were used.

	Oligo name	Oligo sequence (5' → 3')
Cloning	HSPB1 promoter -685, +36 up	agtcgacaggcatgcaccaccatgcccagc
	HSPB1 promoter -685, +36 low	ccatggtggctgactctgctctggacgtctg
	HSPB1 -1098, +36 up	agctgctagccagtggtgagatctcggctc
	XBP1(s) expression up	agctaagcttaccatggtggtggtggcagccgc
	XBP1(s) expression low	agctctcgagttagacactaatcagctggggaa
	Hsp70Ab HSE up	ctagctctattctcgttgcttcgagagagcgcgcctcgaatgttcgcgaaaagagcg
	Hsp70Ab HSE low	gatccgctcttttcgcgaacattcgaggcgcgctctctcgaagcaacgagaatagag
	HSPB1 HSE up	ctagctttccttaacgagagaaggttccagatgagggctgaaccctg
	HSPB1 HSE low	gatccagggttacgccctcatctggaaccttctctcgttaaggaaag
	SERPINH1 HSE up	ctagaggagctggggcgccctccggaagcgtttccaactttccagaagtttctcgggacggc
	SERPINH1 HSE low	tcgagccgtcccgagaaacttctggaaagttggaaacgcttccggagggcgccccagctcct
	BAG3 Δ HSE up	ctagagattatagccgatgactcagggcggagctccgcatccaaccccgggccgcggccaacttctc
	BAG3 Δ HSE low	tcgagagaagttggccgcggcccggggttggatgcggagctccgccctgagtcatcggctataatct
	BAG3 HSE up	ctagagattatagccgatgactcagggcggagctccgcatccaaccccgggccgcggccaacttctctggactggac-cagaagtttctagccggccc
	BAG3 HSE low	tcgagggccggctagaaacttctggtccagtccagagaagttggccgcggcccggggttggatgcggagctc-cgccctgagtcatcggctataatct
ChIP	HSPA5 ChIP fwd	tgcctcggggtcagaagtcgc
	HSPA5 ChIP rev	caggtctagaaatacaggccgc
	SERPINH1 ChIP fwd	agggaggcagtaggacccag
	SERPINH1 ChIP rev	cgtgactctcgctctcgagc
	BAG3 ChIP fwd	gattatagccgatgactcagggcg
	BAG3 ChIP rev	agtgtctggaaatagcctcc
	HSPA1A ChIP fwd	ctctggagagttctgagcag
	HSPA1A ChIP rev	tataagtcgtcacggagacc
	DNAJB1 ChIP fwd	cgatgtcgcgtgtcgctgaa
	DNAJB1 ChIP rev	cgaccagtcccggactctata
QPCR	GAPDH_QPCR_fwd	gcagctgaaagaagcccaagt
	GAPDH_QPCR_rev	tgtcttccatgccaattgca
	HSPA1A_QPCR_fwd	ccgagaaggacgagtttgag
	HSPA1A_QPCR_rev	acaaaaacagcaatcttggaaagg
	XBP1_QPCR_fwd	ctggaacagcaagtggtaga
	XBP1_QPCR_rev	tctacccagaaggacccagt
	DNAJB1_QPCR_fwd	ttccccagacatcaagaacc
	DNAJB1_QPCR_rev	accctctcatggtccacaac
	DNAJB9_QPCR_fwd	tggtggttccagtagacaaagg
	DNAJB9_QPCR_rev	cttcgttgagtgacagtcctgc
	BAG3_QPCR_fwd	ctccattccggtgatacacga
	BAG3_QPCR_rev	tggtgggtctggtactccc
	HSPA5_QPCR_fwd	ggccgcacgtggaatgac
	HSPA5_QPCR_rev	tccaatatcaacttgaatgtatgg
	HSPB1_QPCR_fwd	cgcgctcagccggcaactc
	HSPB1_QPCR_rev	agccatgctcgtcctgccgc
	SERPINH1_QPCR_fwd	ttgagttggacacagatg
	SERPINH1_QPCR_rev	gcactaggaagatgaagg
	ST13_QPCR_fwd	agaagttcaacctagggcacaga
	ST13_QPCR_rev	ttgatctctcgctcttcacgttt

change (Fig. 1A, C; Fig. S1), nor was the DNAJB1 mRNA level affected (Fig. 1B). Exogenous expression of XBP1s also inhibited the heat shock induced increase in HSPB1 protein and mRNA levels (Fig. 1B, C). XBP1s had an inhibitory effect on the heat shock induced increase in the HSPA1A mRNA level as well, although no effect on the protein level was seen (Fig. 1B, S1). The heat shock induced increase in both DNAJB1 mRNA and protein levels was not affected by XBP1s expression (Fig. 1B, C).

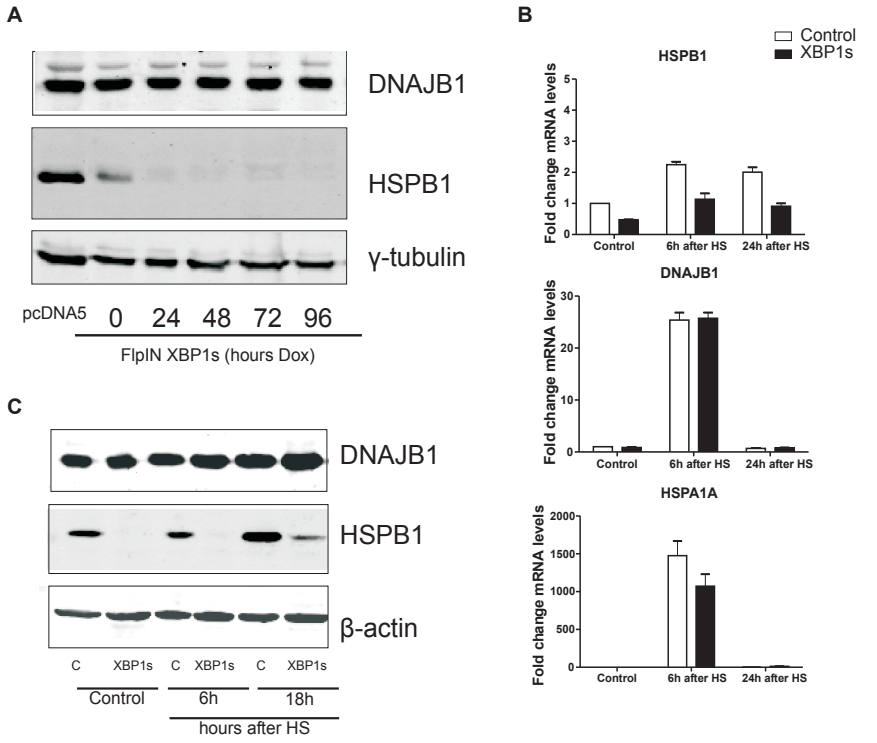

Figure 1 HSPB1 expression strongly decreased in presence of XBP1s.
(A) (Co-)chaperone levels in presence of XBP1s. HEK-cDNA5 and HEK-XBP1s cells were cultured in the presence or absence of doxycycline. Cells were exposed to doxycycline for different periods of time. Cell lysates were subjected to SDS-PAGE and levels of DNAJB1, and HSPB1 were determined by western blotting. γ-tubulin was used as a loading control.
(B) HEK-XBP1s cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. (C) Heat shock response of DNAJB1 and HSPB1 in the presence of XBP1s. HEK-cDNA5 and HEK-XBP1s cells were cultured in the presence or absence of doxycycline and exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of DNAJB1 and HSPB1 were determined by western blotting. β-actin was used as a loading control.

These data led us to examine the effect of exogenous expression of XBP1s on the activity of HSPB1 promoter. We have previously shown that the heat shock induced activity of the HSPB1 gene, as evidenced by that of HSPB1 promoter reporter constructs, is late relative to that of the HSPA1A promoter constructs, being higher in cells that had recovered overnight from a heat shock than in cells 6 hours after heat shock [15]. We thus assayed the activity of a set of HSBP1-luciferase reporter genes in cells that had recovered

for overnight 18 hrs. The activity of all these constructs was strongly inhibited by exogenous expression of XBP1s (Fig. 2A), and the effect of XBP1s on the activity of the reporter genes in heat shocked cells thus mimics its effect on endogenous HSPB1 mRNA levels (Fig. 1B). Note however, that in unstressed cells XBP1s activated the HSPB1 promoter constructs (Fig 2A), while, in contrast, endogenous HSPB1 mRNA levels went down (Fig. 1B). The HSPB1 gene is a somewhat unusual heat shock gene in that it is also constitutively expressed in some tissues. The HSPB1 promoter also has only a single, imperfect HSE (see also Table 1) and for the heat shock induced activity of this promoter HSF1 may be necessary but not sufficient. We thus tested the effect of exogenous expression of XBP1s on reporter gene constructs driven by the Drosophila melanogaster (dm)Hsp70Ab promoter, of which the activity is thought to be solely dependent upon the HSF1 HSE interaction. As shown in Fig. 2B, the activity of the dmHsp70Ab promoter also decreased in presence of XBP1s. We also tested a human HSPA1A promoter construct but found no effect of XBP1s (data not shown). However, the human HSPA1A promoter construct has a high activity in non-stressed cells and the increase in activity as a result of heat shock is relatively slight. In contrast, the dmHsp70Ab promoter construct is virtually silent in non-stressed cells. The high background of the HSPA1A promoter construct may obscure the effect of XBP1s that is seen on the endogenous HSPA1A mRNA level (Fig. 1B).

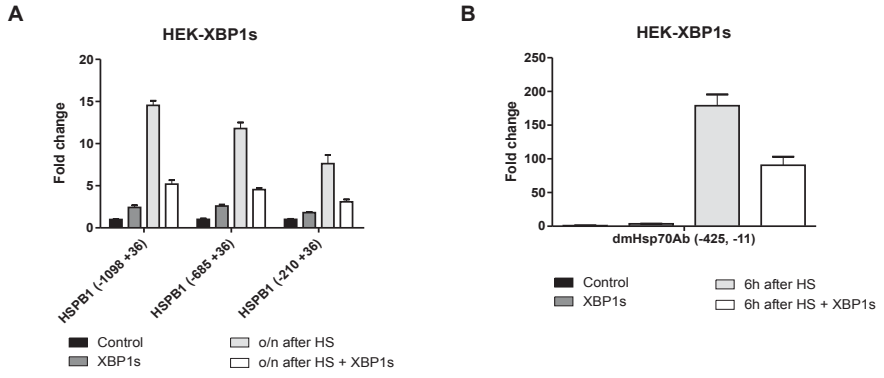

Figure 2 Heat shock response of two heat shock protein promoters in presence of XBP1s (A-B) The effect of XBP1s expression on the activity of HSPB1 (A) and dmHsp70Ab promoter constructs (B). HEK-XBP1s were transfected with a mixture of reporter vector and β-actin-β-galactosidase (9:1 ratio). At 24 hours after transfection, the expression of XBP1(s) was induced by adding 0.1 µg/ml doxycycline. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated periods of time and harvested. Promoter activities were determined by dividing firefly luciferase values by β-galactosidase values to correct for varying transfection efficiencies. The results are the average of four independent transfections (standard deviations are indicated by error bars).

In order to map the interaction site of XBP1s on a promoter, we need to use reporter constructs. As shown above, the two promoter constructs that report an effect of XBP1s are the HSPB1 and the dmHsp70Ab reporter constructs. To confirm that the activity of these constructs is sensitive to XBP1s, we turned to T-REx HEK293 expressing a dominant negative IRE1α mutant. In these cells, XBP1 mRNA cannot be spliced and these cells thus lack XBP1s. In heat shocked dnIRE1α expressing cells the activity of the dmHsp70Ab and the HSPB1 promoter constructs was higher than in control cells (Fig. 3A), again suggesting that XBP1s inhibits the activity of these promoters.

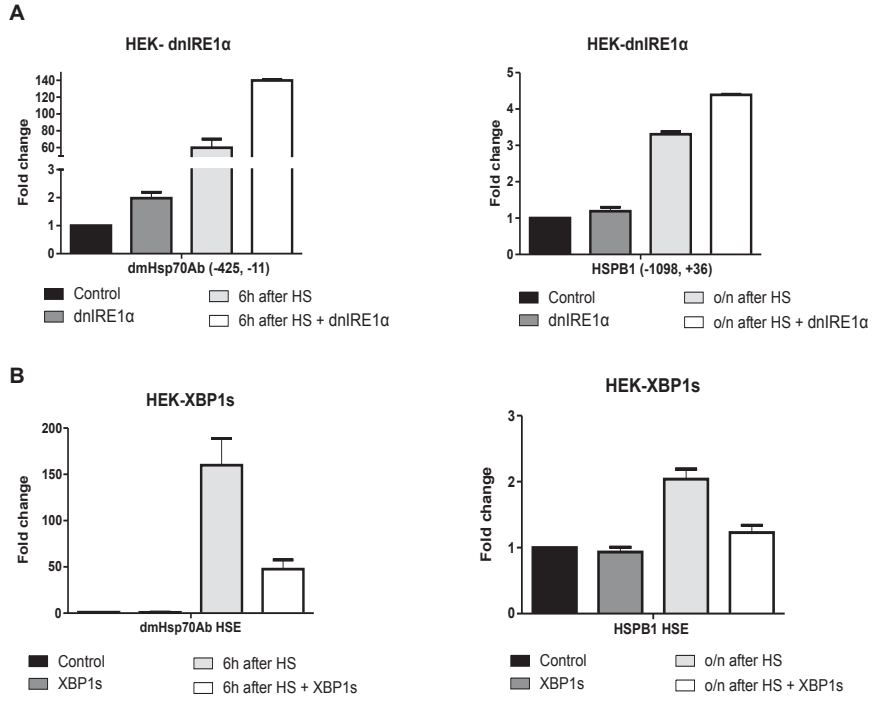

Figure 3 XBP1s sensitivity of two heat shock promoters.
(A) The effect of dnIRE1α expression on the activity of the HSPB1 and the Hsp70Ab promoter constructs. HEK-dnIRE1α cells were transfected with a mixture of reporter vector and β-actin-β-galactosidase (9:1 ratio). At 24 hours after transfection, the expression of dnIRE1α was induced by adding 0.1 μg/ml doxycycline. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated periods of time and harvested. Relative luciferase activities were calculated as detailed in the legend to Fig. 2.
(B) The effect of XBP1s expression on the heat shock response of two HSE containing constructs. HEK-XBP1s cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection, the expression of XBP1s was induced by adding 0.1 μg/ml doxycycline. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated periods of time and harvested. Relative luciferase activities were calculated as detailed in the legend to Fig. 2.

The regulatory element shared by these promoters is of course the HSE. To test whether XBP1s acts via the region containing the HSE, we cloned a 50 bp sequence containing the two dmHsp70Ab HSEs and a 41 bp sequence containing the single HSPB1 HSE in front of the SV40 promoter (see Table 1 for the sequence of these regions). The dmHsp70Ab HSE construct was nicely responsive to a heat shock and its activity was strongly inhibited by exogenous XBP1s (Fig. 3B). The HSPB1 promoter contains a single, not quite perfect, HSE and the activity of the construct containing this HSE was increased only two fold in heat shocked cells. The activity was again inhibited by exogenous XBP1s (Fig. 3B). These data show that the region containing the HSE is a target of XBP1s. We could not detect a consensus XBP1 binding site (CCACG or ACGT; [19, 20]) in the dmHsp70Ab or the HSPB1 HSE regions nor do these regions shown any sequence similarity besides the HSE (GAAnnTTCnnGAA) [21]. XBP1s thus appears to act either directly or indirectly via the HSE.

Possible common HSF1 and XBP1 target genes.

The data reported above show that at least some HSF1 target genes are also targets of XBP1s. In an effort to identify other common HSF1 and XBP1s target genes systematically, we compared the ChIP-on-chip data identifying mouse XBP1s binding sites [19] with the ChIP-on-chip data pinpointing human HSF1 targets ([22], [23]) yielding 12 candidate genes. We then looked whether the sequence containing the potential mouse XBP1s binding site was conserved in the promoter region of the corresponding human gene and whether this region contained or was close to a predicted HSE. Four human genes met these criteria: BAG3, SERPINH1, ST13 and UBB (Fig. S2). We selected BAG3 and SERPINH1 for further study. In both the human and the mouse BAG3 genes the putative XBP1 binding region is located around the transcription initiation site and a putative HSE is located just downstream in the 5' UTR (Fig. S2). In the SERPINH1 gene the putative HSE is within the putative XBP1 binding region. The transcription initiation sites of the human and mouse genes have been mapped upstream of the putative XBP1 binding region which places the sequence in the 5' UTR (Fig. S2). (see Fig. S2). We did not look further at the ST13 gene as it was not heat shock responsive (Fig. S1 and data not shown), although the promoter region does contain a functional HSE ([16]), or at the UBB gene as it is not known as a typical HSF1 target. Note that none of these putative XBP1 binding sites contain a consensus XBP1 site [19].

We first tested whether the transcript levels of BAG3 and SERPINH1 were influenced by expression of XBP1s. As shown in Fig. 4A, the level of BAG3 and SERPINH1 mRNAs increased in heat shocked XBP1s expressing cells. The level of BAG3 mRNA, but not that of SERRPINH1 mRNA, also increased in non stressed cells. We could not detect a marked increase in BAG3 protein in XBP1s expressing cells (Fig. S1).

To determine whether the putative BAG3 and SERPINH1 XBP1 binding sites

and HSE's could be involved in this effect of XBP1s, we cloned the relevant sequences in front of the SV40 promoter in the pGL3 promoter vector and tested the effect of XBP1s expression and/or a heat shock on the activity of the resulting reporter genes. Both constructs were poorly active in non stressed cells. Expression of XBP1s increased activity. However, at least in the case of the BAG3 construct, this increased activity is mediated by the region containing the HSE, and not by that containing the putative XBP1s binding site, as deletion of the HSE abolished activity. In heat shocked cells both constructs were quite active, showing that they indeed contain HSE's (Fig. 4B). Deleting the putative HSE from, but retaining the putative XBP1 binding region in, the BAG3 reporter construct led to a loss of the heat shock induced activity. As in the case of the constructs containing either the

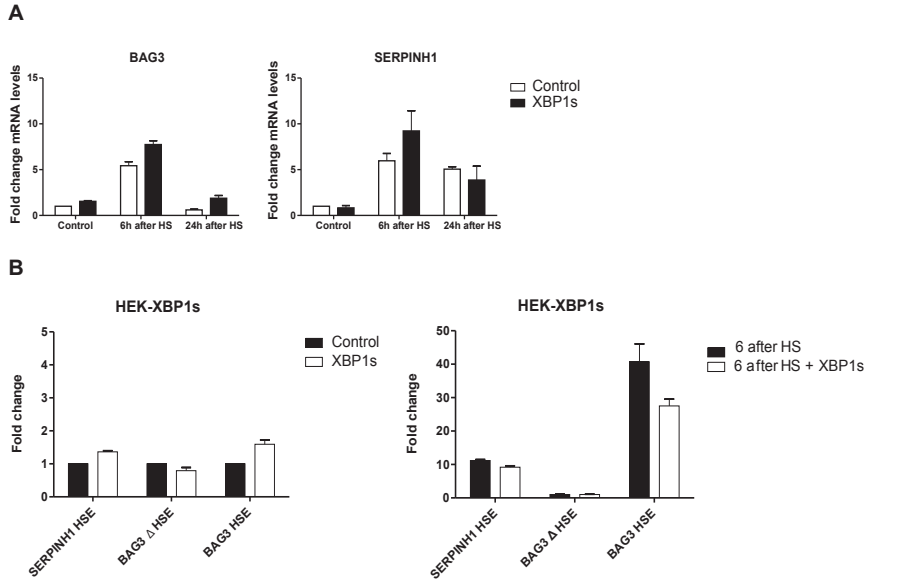

Figure 4 XBP1 sensitivity of several HSE's.

(A) Relative changes in transcript levels of the BAG3 and SERPINH1 genes in stressed or non-stressed cells with or without XBP1s overexpression. HEK-XBP1s cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells.

(B) The effect of XBP1s expression on the activity of several HSE containing constructs. HEK-XBP1s cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection, the expression of XBP1s was induced by adding 0.1 µg/ml doxycycline. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated periods of time and harvested. Relative luciferase activities were calculated as detailed in the legend to Fig. 2.

The left panel shows the effect of XBP1s on the activity of the HSE containing constructs before stress; the right panel that in heat shocked cells.

dmHsp70Ab HSE or the HSPB1 HSE regions (Fig. 3B), the activity of the BAG3 HSE construct was inhibited by exogenous expression of XBP1s (Fig. 4B). The effect of XBP1s on the SERPINH1 construct was only marginally inhibitory.

Binding of exogenous XBP1s to HSF1 target promoter regions.
The data presented above indicate that exogenous expression of XBP1s does affect the expression level of HSF1 target genes. We thus attempted to show that XBP1s binds to the regions containing the HSE and/or affects the binding of HSF1 to the HSE. In electromobility shift assays (EMSA's) we could not detect XBP1s binding to the putative XBP1s binding or interaction sites in the BAG3, HSPB1 or dmHsp70Ab promoters. However, this is probably an experimental problem as we could also not detect an interaction of XBP1s with a known target site (data not shown). The HSE sequences did interact with HSF1 as evidenced by the bandshift using extracts from heat shocked cells; bands which could be supershifted with antiHSF1 (data not shown). However, the intensities or the mobilities of the bandshifts were not affected by the presence of XBP1s, indicating that XBP1s does not affect the binding of HSF1 to the selected HSEs as measured in this assay.
In unstressed cells overexpressing XBP1s, binding of XBP1s to the putative XBP1s binding sites in the promoter regions of the BAG3 and SERPINH-1genes as well as to the known XBP1s binding site in the promoter of the HSPA5 gene, a known XBP1s target gene (Fig. S1), could be detected by ChIP (Fig. 5A). The percentage recovery is low (compare for example the ChIP data obtained with HSF1 shown in Fig. 5B) but this could be a technical problem due to inefficient precipitation by the XBP1 antibody. We did not detect significant binding of XBP1s to the HSPA1A or DNAJB1 promoter regions in unstressed cells (Fig. 4A). Unfortunately we have been unable to recover regions from the HSPB1 promoter in ChIP assays. In cells that had recovered from a heat shock for 6 hours (Fig. 5A, 6h after HS), XBP1s binding was always less than in non stressed cells or in cells that had recovered after 24 hours, suggesting that XBP1s might lose its DNA binding activity during heat shock.
In unstressed cells there is very little binding of HSF1 to its target promoter regions as assessed by ChIP. In stressed cells a marked increase in HSF1 binding is seen 6h after heat shock; this binding is lost again after 24 hrs after heat shock as expected from the transient activation of HSF1 (Fig. 5B). There appeared to be some effect of XBP1s on HSF1 binding in heat shocked cells: the extent of binding of HSF1 to the BAG3 and SERPINH1 HSEs increased, while that to the HSEs of the HSPA1A and DNAJB1 promoters decreased. At first glance, this seems to agree with the increase in BAG3 and SERPINH1 mRNA levels, and the decrease in HSPA1A mRNA level. However, we did not see an effect of XBP1s on the level of the DNAJB1 transcript in heat shocked cells, while HSF1 binding to this promoter also decreased in the presence of XBPs.

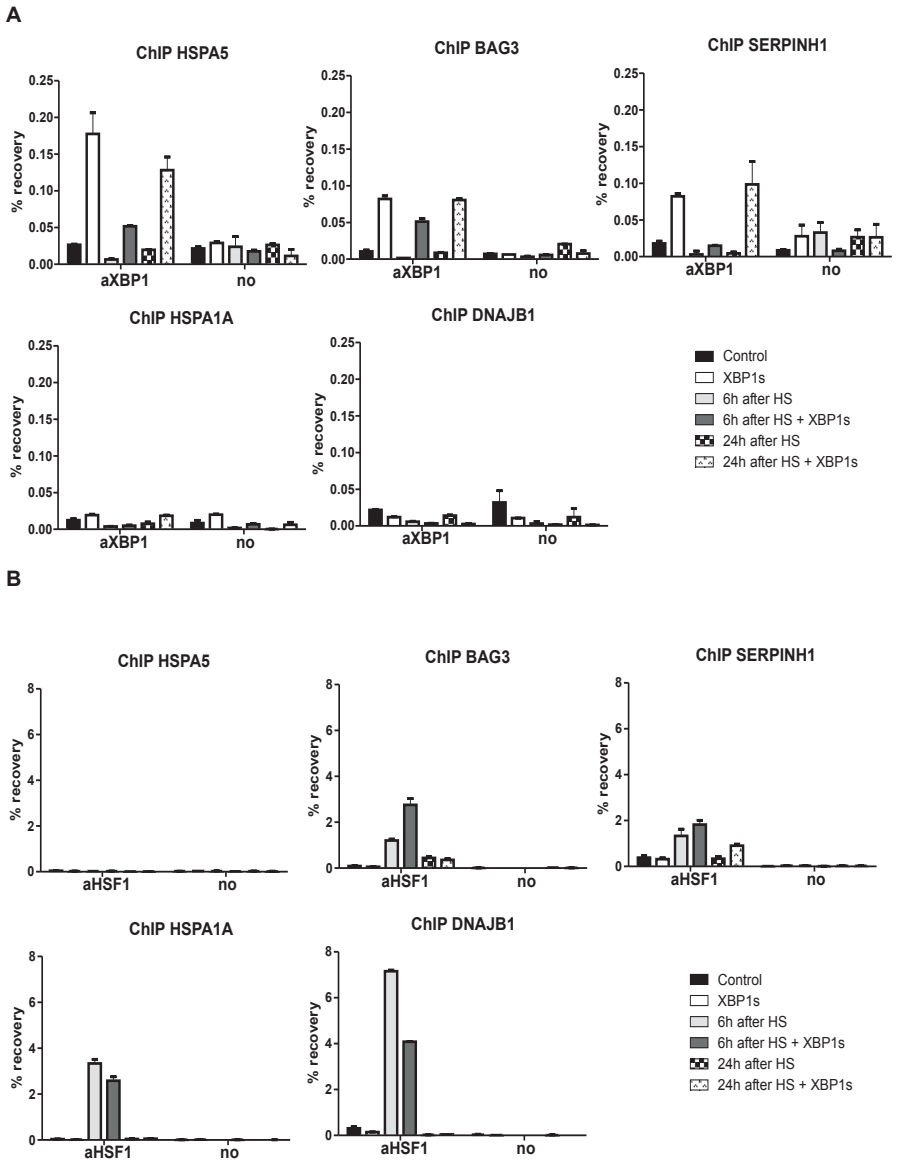

Figure 5 ChIP analysis of XBP1 and HSF1 binding.
(A) Chromatin immunoprecipitation (ChIP) using nuclear extracts from control and XBP1s expressing cells was performed with an XBP1 antibody or no antibody added. Bound chromatin was analyzed by QPCR using a primer set surrounding the HSE of the BAG3, SERPINH1, HSPA1A or DNAJB1 promoter. A primer set for the HSPA5 promoter was used as well. Cells were either non-stressed or harvested 6 hrs or 24 hrs after heat shock, as indicated.
(B) As in (A) except that an antibody against HSF1 instead of XBP1 was used.

Discussion

Together our data suggest that XBP1s can modulate the heat shock response and inhibits the expression of at least dmHsp70Ab and HSPB1 while enhancing that of BAG3 and SERPINH1. The mechanism by which XBP1s does affect the heat shock response of these genes remains to be resolved. Our data point to the HSE as the common target of XBP1s and HSF1. However, the effect of XBP1s on HSE containing promoter constructs was always activating in non-stressed cells and inhibitory in heat shocked cells, while the response of the endogenous genes was either negative (HSPB1, HSPA1A) or positive (BAG3, SERPINH1). Furthermore, we could not detect XBP1s binding sites in these regions even though in ChIP-on-chip experiments at least some of these regions were identified as XBP1s binding sites; a finding confirmed by our ChIP data. There is also some discrepancy between the DNA binding data and the expression data. According to our ChIP data, XBP1s binding is lost from the HSPA5 promoter in heat shock cells, suggesting that XBP1s is inactivated in heat shocked cells, possibly by sumoylation [24], a post-transcriptional modification that also inactivates HSF1 [25]. Alternatively, XBP1s could be deacetyled and thus be less active as well as less stable [26]. An inactivation of XBP1s in heat shocked cells would be in agreement with our previous results showing that, although a heat shock does activate the UPR, the downstream UPR target genes are not activated; these are activated in a later stage by other transcription factors [15]. We thus cannot exclude the possibility that the effect of XBP1s is indirect. For example, it has been recently demonstrated that XBP1s enhances the proteosomal degradation of the transcription factor FoxO1[27]. The experimental set-up used here is artificial in that we overexpressed XBP1s continuously, while under physiological conditions XBP1s expression in heat shocked cells is only transient and the effect of XBP1s may then be marginal. However, in some, mostly secretory, cell types XBP1s is expressed at high levels and in those cells the effect of XBP1s on HSF1 target genes could be marked. In particular, our experiments predict an inverse relation between expression of HSPB1 and XBP1s which might provide an explanation for the pro-apoptotic phenotype linked with XBP1s overexpression in certain cell-types. This prediction remains to be tested.

Acknowledgements

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Supplemental tables and figures

S1

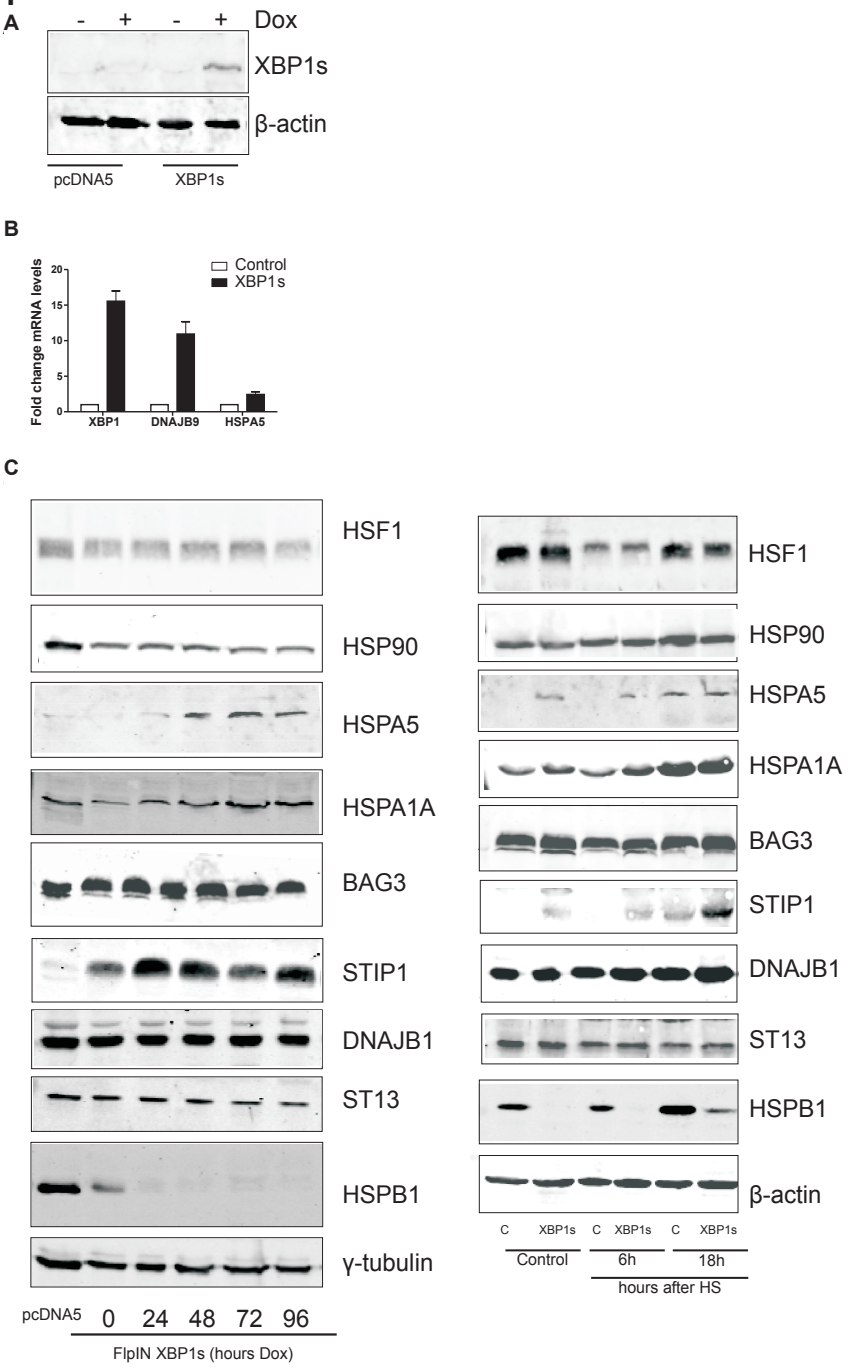
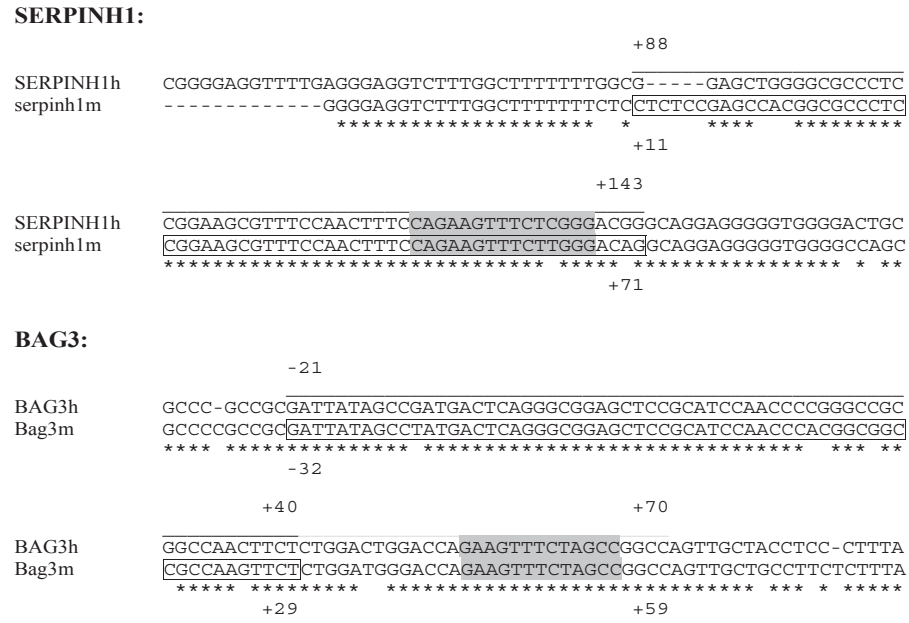


Figure S1: Characterization HEK-XBP1s cell-line.
(A) XBP1s expression levels before or after treatment with doxycycline were determined using SDS-PAGE followed by western blotting. β-actin was used as a loading control.
(B) Relative changes in transcript levels of various genes in XBP1s expressing cells. HEK-XBP1s cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells.
(C) Effect of XBP1s expression and/or heat shock on the level of HSF1 and of several (co)-chaperones. Left panel: HEK-cDNA5 and HEK-XBP1s cells were cultured in the presence or absence of doxycycline. Cells were exposed to doxycycline for different periods of time. Cell lysates were subjected to SDS-PAGE and levels of the (co)-chaperones as well as HSF1 as indicated were determined by western blotting. γ-tubulin was used as a loading control. Right panel: HEK-cDNA5 and HEK-XBP1s cells were cultured in the presence or absence of doxycycline and exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of several (co)-chaperones and HSF1were determined by western blotting. β-actin was used as a loading control.

S2



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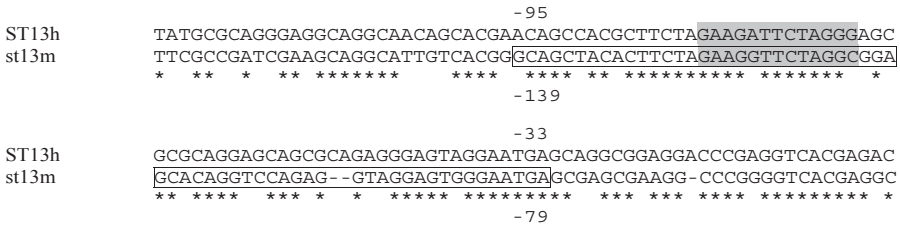

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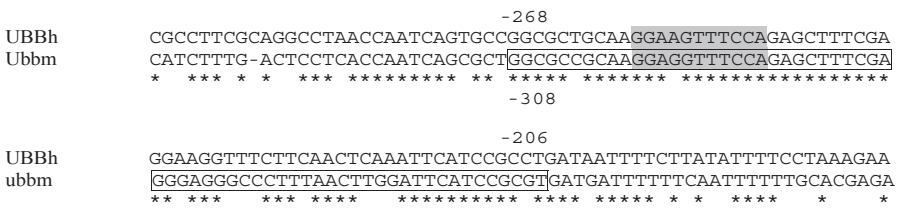

Figure S2: Genomic orientation HSE's SERPINH1, BAG3, ST13 and UBB.
Mouse genes with XBP1s binding sites identified by ChIP-on-chip analysis [19] were compared with the ChIP-on-chip data pinpointing human HSF1 targets [22], [23]. The sequence containing the potential mouse XBP1s binding site was checked for conservation in the promoter region of the corresponding human gene. Shown is the alignment of the mouse (Mus musculus, m) and human (Homo sapiens, h) sequences of the promoter regions of the genes indicated. The numbering is relative to the transcription initiation site as predicted from the RefSeq mRNA sequences (www.ncbi.nlm.nih.gov). The sequence of the putative XBP1 binding region is boxed; the sequence of the predicted HSE is marked in gray.
The black lines above the human sequences indicate the region which was cloned into the pGL3-promoter. The sequence covered by the grey line above the human BAG3 sequence is not included in the pGL3-promoter- BAG3 Δ HSE construct, while it is included in the pGL3-promoter- BAG3 construct.

Chapter 4

Co-chaperones are limiting in a depleted chaperone network

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Abstract

To probe the limiting nodes in the chaperoning network which maintains cellular proteostasis, we expressed a dominant negative mutant of heat shock factor 1 (dnHSF1), the regulator of the cytoplasmic proteotoxic stress response. Microarray analysis of non stressed dnHSF1 cells showed a two- or more fold decrease in the transcript level of 10 genes, amongst which the (co-)chaperone genes HSP90AA1, HSPA6, DNAJB1 and HSPB1. Glucocorticoid signalling, which requires the Hsp70 and the Hsp90 folding machines, was severely impaired by dnHSF1, but fully rescued by expression of DNAJA1 or DNAJB1, and partially by ST13. Expression of DNAJB6, DNAJB8, HSPA1A, HSPB1, HSPB8 or STIP1 had no effect while HSP90AA1 even inhibited. PTGES3 (p23) inhibited only in control cells. Our results suggest that the DNAJ co-chaperones in particular become limiting in a depleted chaperoning network. Our results also suggest a difference between the transcriptomes of cells lacking HSF1 and transcriptomes of cells lacking HSF1 and cells expressing dnHSF1.

Introduction

All cells contain an extensive network of chaperones which together maintain proteostasis, i.e. this network aids in the folding of the primary peptide chain, the refolding of unfolding proteins and the removal of misfolded proteins (for reviews, see [1-8]). Two of the major nodes in the network are the Hsp70 and Hsp90 folding machines. At the core of these machines are Hsp90 and Hsp70, the proteins that promote folding; the activity and substrate specificity is controlled by a number of co-factors and co-chaperones. For Hsp70 it is the DNAJ (Hsp40) proteins that determine substrate specificity. DNAJs also stimulate ATP hydrolysis by Hsp70. The human genome contains over 40 DNAJ genes [9-11]. Some of these are highly tissue specific, others may be dedicated to a particular substrate or cooperate only with a specific Hsp70 and some may be redundant [12].

The diversity of DNAJs does show that these are important determinants of the activity and specificity of the Hsp70 folding machine. The chaperoning capacity of the cell is enhanced by additional chaperone synthesis as part of a proteotoxic stress response, either the heat shock response in the case of cytoplasmic stress or the unfolded protein response in the case of ER stress. That an increase in chaperones is required to combat proteotoxic stress suggests that under normal conditions the chaperone capacity of a cell is limiting. Indeed, exogenous expression of aggregation-prone proteins, such as proteins with an expanded glutamine tract (polyQ), is toxic unless chaperones are also over-expressed [13-17]. Cytoplasmic proteotoxic stress signals to heat shock factor 1 (HSF1), which then activates the transcription of a number of genes encoding a variety of chaperones, together known as the heat shock proteins. In the absence of stress, HSF1 is generally believed to be kept inactive in the cell by direct interaction with Hsp90, p23 and immunophilins (for reviews, see [18-20]). HSF1 null mice show the expected stress-related phenotypes, such as a complete lack of the heat shock response and the inability to develop thermotolerance. However, they also suffer from neuronal, developmental and germ cell defects [21-26], which cannot be directly linked to the heat shock response and which strongly suggests that HSF1 also regulates gene expression under non-stress conditions. Microarray analysis resulted in the identification of 49 genes (19 related to immune response) that are expressed at reduced levels in HSF1 null fibroblasts compared with wild type cells cultured under physiological conditions. The immune response of HSF1 null mice was shown to be severely impaired [27]. More recently, direct evidence for the stress independent regulation of genes by HSF1 was provided in the case of the multi-drug resistance gene 1 [28], and the IL-6 gene [29]. Furthermore HSF1 inhibits heregulin induced transcription in breast carcinoma cells [30].

A number of studies have shown that the quality of the heat shock response diminishes with aging [31-37], a decrease that may be the result of a decrease in the activity of the deacetylase SIRT1 [38]. Senescence of cultured

human fibroblasts is accompanied with a diminishing heat shock response and a reduction in the affinity of HSF1 for the heat shock element (HSE; [34]). Aging-related failure of HSF1 will interfere with an organisms' ability to combat cellular stress and increase the susceptibility to protein folding disease [6, 8, 13, 14, 39-41]. Moreover, with accumulating evidence showing that HSF1 also regulates gene expression under non-stress conditions (see above), its decline may already cause phenotypic defects in the absence of exogenous stress [3, 4].

Here we have used a dominant negative HSF1 mutant to inhibit HSF1 activity. As expected, a number of chaperone and co-chaperone genes were downregulated by dnHSF1. To test which (co-)chaperone is limiting in dnHSF1 expressing and thus chaperone depleted cells, we used the glucorticoid response to probe the chaperoning network. Maturation of the steroid hormone receptor is known to be controlled by both the Hsp70 and the Hsp90 folding machinery (for review, see [42]) and augmenting the chaperone network by either stress [43] or expression of a constitutively active HSF1 mutant [44] potentiates the glucocorticoid response. We show here that it is, unexpectedly, primarily the DNAJ (Hsp40) proteins which become limiting when the chaperoning network is depleted.

Materials and Methods

Recombinant DNA constructs

Oligonucleotides that were used to generate recombinant DNA constructs are listed in Table 1. Plasmid pLmHSF1SN that contains the code for the HSF448 mutant was kindly donated by Dr. Wang [45]. The 1.36-kb *Xho*I fragment of pLmHSF1SN was cloned into pcDNA5-FRT/TO (Invitrogen), resulting in plasmid pcDNA5-HSF448. The code for the HSF1 mutant HSF379 was PCR amplified from pLmHSF1SN using the HSF379 primer set and cloned into the *Hin*dIII and *Xho*I sites of pcDNA5-FRT/TO, yielding plasmid pcDNA5-HSF379 (dnHSF1). The promoter constructs pGL3-HspB1 (-685/+36), pGL3-DNAJA1 (-464/+167), pGL3-DNAJB1 (-508/+38), pGL3-Hsp90AA1 (-188/+18), pGL3-ST13 (-400/+141), pGL3-STIP1 (-1264/+145), pGL3-PTGES3 (-1108/+104), pGL3-RMB23 (-1265/+189), pGL3-PMVK (-1183/+147), pGL3-BiP (-2742/+202), pGL3-CHOP (-936/+2), and pGL3-HSPA1A (-313/+196) were made by PCR amplifying the promoter fragments from human genomic DNA using the respective "prom" primer sets and cloning the fragments into pGL3-Basic (Promega). The expression plasmids pcDNA5-HSPB1, pcDNA5-HSPB8, pcDNA5-ST13, pcDNA5-STIP1, and pcDNA5-PTGES3 were made by PCR amplifying the cDNAs from HEK293 RNA using the respective "exp" primer sets and cloning the cDNAs into pcDNA5-FRT/TO. Expression plasmids pcDNA5-V5-DNAJA1, pcDNA5-V5-DNAJB1, pcDNA5-V5-DNAJB6, and pcDNA5-V5-DNAJB8 were kindly donated by J. Hageman (University of Groningen, The Netherlands; [46]).

Expression construct pCMV-SPORT6-Hsp90AA1 was obtained from Imagenes (www.imagenes-bio.de). The Hsp90AA1 coding sequence was completed at the 5' end by inserting the corresponding fragment PCR amplified from human cDNA *SacII-MscI*. Plasmid pOTB7-STIP1 was obtained from Imagenes. The *Eco*RI (blunt) - *Xho*I fragment of pOTB7-STIP1 was cloned into the *Hin*dIII (blunt) and *Xho*I sites of pcDNA5-FRT/TO, resulting in plasmid pcDNA5-STIP1. The glucocorticoid-responsive reporter plasmid pGRE-Luc was made by annealing the GRE primer set and cloning the double stranded oligo into the *Nhe*I and *Bgl*II sites of pGL3-promoter (Promega). The *Drosophila melanogaster* Hsp70-luciferase reporter construct pHL and the Hsp70 expression construct were described earlier [47]. Plasmid pRL-CMV was obtained from Promega. All plasmid constructs were sequence verified.

Tissue culture, transfections, and reporter gene assays

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-HSF448, HEK-HSF379 and HEK-cDNA5 that carry a single copy of the tetracycline inducible plasmids pcDNA5-HSF448, pcDNA5-HSF379, and pcDNA5-FRT/TO, respectively. The cells were cultured at 37°C / 5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with ~ 0.2 µg plasmid per well. For testing the heat shock response in stable HEK293 cell lines, cells were transfected with 160 ng pHL, and 40 ng pCMV-RL. At 48 h after transfection, cells were either left at 37ºC/ 5% CO_2 (control) or incubated at 45ºC for 30' (heat shock). After 6 h recovery at 37ºC/ 5% CO_2, cells were harvested for reporter gene analysis. For analysis of promoter activities, cells were transfected with a mixture of 160 ng luciferase reporter plasmid and 40 ng β-actin-β-galactosidase or pCMV-RL per well. For testing glucocorticoid responsiveness, the culture medium of the cells was first replaced with medium supplemented with 10% steroid-free fetal calf serum (Hyclone), and then the cells were transfected with a mixture of 150 ng pGRE-Luc and 50 ng β-actin-β−galactosidase per well. At 24 h after transfection, the culture medium was replaced with medium containing varying concentrations of dexamethasone (Centrafarm). At 48 h after transfection cells were lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 40 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus (Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence

was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 40 µl cell lysate was mixed with 50 µl luciferin solution and luminescence was again measured with the Lumat luminometer. All reporter gene assays were performed in triplo.

RNA isolation and microarray analysis

HEK-HSF379 or HEK-cDNA5 cells were either left untreated or treated with doxycyclin for 48 hours. Total RNA was isolated using Trizol according to the manufacturer's instructions (Invitrogen) and copied into Cy3-labeled (untreated cells) or Cy5-labeled (doxycycline treated cells) cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS, or the reverse for the repeat array. Labeled cRNA samples were hybridized to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). Only genes that passed the GeneSpringGX standard quality control criteria (free trial available at www.genespring.com) were included in the analysis. We used a cut-off level of 2-fold changed expression (average signal intensity across the array) and an arbitrarily chosen signal cut-off of > 50.

Western blot analysis

Cell pellets were homogenized in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4X sample buffer (200 mM Tris-HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% Glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. For detection of eIF2α phosphorylation, samples were prepared as described [48]. Protein samples were separated in 12% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1: 15,000 dilution, Hsp70 antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal DNAJB1 antibody (anti-Hsp40; SPA-400; Stressgen) at a 1:10,000 dilution, monoclonal Hsp90 antibody (610418, BD Biosciences) at a 1:1,000 dilution, HSPB1 antibody, obtained from dr. A. Zantema, at a dilution of 1:400, monoclonal eIF2α antibody was at a 1:500 dilution, polyclonal phosphorylated eIF2α antibody (E2152; Sigma) was used at a 1:1,000 dilution, monoclonal V5 antibody (R96025; Invitrogen) was used at a 1:5,000 dilution, polyclonal ST13 antibody (ab13490; Abcam) at a 1:1,000 dilution, polyclonal STIP1 antibody (ab65046; Abcam) a 1:1,000 dilution, monoclonal p23 antibody (ab2814; Abcam) at a 1:1,000 dilution, polyclonal HSPB8 antibody, obtained from dr. W. Boelens, at a dilution of 1:1,000, and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary

antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye™ 680 conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32220 respectively, LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

Table 1 Oligonucleotides that were used to generate recombinant DNA constructs

Oligo name	Oligo sequence (5' → 3')	Oligo name	Oligo sequence (5' → 3')
HSF379-for	agctaagcttaccatggatctgcccgtgggcc	BiPprom-for	tctcgaggtatttttagtagagactgggcac
HSF379-rev	agctctcgagctacaggcaggctacgctgaggc	BiPprom-rev	accatggtgccagccagttgggcagcag
PMVKprom-for	agctaagcttactcaggtaaaacaggagatgtg	HSP90prom-for	agctaagcttgcgcaggcgctgttcctgg
PMVKprom-rev	agctccatggccaaacagatatggggagaaaag	HSP90prom-rev	agctccatggcgcccggaggccacaccc
RBM23prom-for	agctctcgagtatccaagacccaaaggggcc	HSPA1Aprom-for	aagatcttgaagcgcaggcggtcagca
RBM23prom-rev	agctccatggcagttccgggtccccgcag	HSPA1Aprom-rev	aaagcttccggttccctgctctctgtc
STIP1prom-for	agctaagcttgtggggcaggtggaattaaag	HSP90AA1exp-for	tccgcggtcacttagccaagatgcctg
STIP1prom -rev	agctccatggcgcagcgcggtccggaacc	HSP90AA1exp-rev	tggccaatcatagagatatctgcacc
HSPB1prom -for	agtcgacaggcatgcaccaccatgcccagc	HSPB1exp-for	agctaagcttaccatgaccgagcgccgcgtc
HSPB1prom -rev	accatggtggctgactctgctctggacgtctg	HSPB1exp-rev	agctctcgagttacttggcggcagtctcatcg
ST13prom -for	agctaagcttccccttccggcggaggcg	HSPB8exp-for	agctaagcttaccatggctgacggtcagatg
ST13prom -rev	agctccatggtagggaggtggtgg	HSPB8exp-rev	agctctcgagtcaggtacaggtgacttcctggct
PTGES3prom -for	agctaagcttaatacctttagtgcttattatgaagc	ST13exp-for	agctaagcttaccatggaccccccgcaaagtg
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DNAJA1prom-for	agtcgaccacgcgtgaaaaacagcaagac	PTGES3exp-for	agctggatccaccatgcagcctgcttctgcaaagtg
DNAJA1prom-rev	accatggtggctgaggccggtgtgtgaggga	PTGES3exp-rev	agctctcgagttactccagatctggcatttttttc
DNAJB1prom-for	aagtcgaccagacacaggttaggtagttcgtcc	GRE-up	ctagcggtacattttgttctagaacaaaatgtaccggta-catttttgttct
DNAJB1prom-rev	accatggccccctcctgcggcccgccga		
CHOPprom-for	tgagctctgtcacccaggctggagtgc	GRE-low	gatctagaacaaaatgtaccggtacattttgttctagaacaaaat-gtacc
CHOPprom-rev	tagatctctgacctcgggagcgcctggctg		

Results

Dominant negative HSF1 mutants.

To block HSF1 signalling in human HEK293 cells we decided to use a dominant negative mutant reasoning that, given the interaction of HSF1 with other cellular components, the effect of a transcriptionally inactive mutant could well be different from the effect of HSF1 being completely absent. Two dominant negative HSF1 mutants containing, respectively, the first 379 (HSF379) and first 448 (HSF448) amino acids have been described (reviewed by [49]). HSF379 lacks both the potent trans-activation domain at the extreme C-terminus and the weaker, more N-terminal, trans-activation domain, whereas HSF448 still has the weak trans-activation domain. The heat shock-mediated induction of endogenous Hsp70 was completely abolished by HSF379, showing its potent dominant-negative activity (Fig. 1). Surprisingly, HSF448 was a very poor inhibitor of heat shock-mediated induction of Hsp70 (data not shown). Moreover, HSF448 caused a significant increase in the basal expression of Hsp70 (Fig. 1). Since this observation was in conflict with earlier data showing the dominant-negative activity of HSF448 [45], we tested the activities of both HSF1 mutants in a luciferase reporter gene assay. As expected, HSF379 completely inhibited the heat shock mediated in-

duction of the *D. melanogaster* Hsp70 promoter (Fig. 2). In the experiments reported below HSF379 was used to inhibit HSF1 activity and will be referred to as dnHSF1.

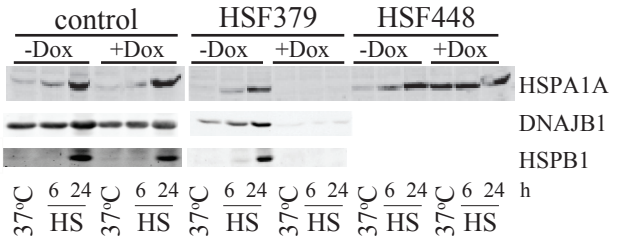


Figure 1 The HSF1 mutants HSF379 and HSF448 have different effects on basal and heat shock-induced Hsp70 expression. Parental Flp-In HEK293 cells and HEK293 cells carrying a stably integrated copy of the pcDNA5-HSF379 (HEK-HSF379) or pcDNA5-HSF448 (HEK-HSF448) plasmid were cultured in the absence or presence of doxycycline. Cells were exposed to a heat shock (30', 45°C), harvested at the indicated time point (h) after heat shock, and subjected to western blot analysis using an anti-Hsp70 antibody.

Transcriptome changes in the presence of dnHSF1.

If HSF1 plays a role even in the absence of exogenous stress, then exogenous expression of a dominant negative HSF1 mutant in unstressed cells should change the transcriptome. We therefore compared the transcriptomes of HEK cells with or without doxycycline and with or without dnHSF1using a two-color 44K Agilent Human Expression Profile Array. The transcripts of only 10 genes showed a more than two fold lower level in the presence of dnHSF1 (Table 2 in bold and Table 3). Four of these, namely HSPA6 (hsp70B'), HSP90AA1 (Hsp90), DNAJB1 (Hsp40) and HSPB1 (Hsp27), encode chaperones (Table 2 in bold). The steady state level of the corresponding proteins was also reduced in dnHSF1 expressing cells (Fig. 3; note that the HSPA6 mRNA level is very low in non-stressed HEK293 cells;[50]). Surprisingly, there was a distinct difference between dnHSF1 expressing cells and mouse embryonic fibroblasts lacking HSF1: the *hsf-/hsf-* MEFs contain wild type levels of Hsp90 and DNAJB1.

The levels of the transcripts of a number of other chaperone genes did not quite meet the "two fold" lower in the presence of dnHSF1 cut-off, but did come close (AHSA2 for example; Table 2). To test whether HSF responsiveness is a general property of genes encoding (co-)chaperones, we looked at the response of all known members of the HSP gene families (HSPH, HSPA, DNAJ and HSPB) as well as other known (co-)chaperones coding genes expressed in HEK 293 cells (Table 2). Of the HSPA (Hsp70) genes, only HSPA6 responded strongly to dnHSF1. Similarly, very few members of the large DNAJ (Hsp40) family were downregulated by HSF1. This is rather surprising as the DNAJ proteins determine the substrate specificity of

and stimulate the activity of the Hsp70 folding machine and are thus critical nodes in the chaperoning network of the cell. Also most of the Hsp70 and Hsp90 co-chaperones are not responsive to dnHSF1. For example, of the 14 Hsp90 co-factors listed in a recent review [51], only the two AHA1 homologs as well as STIP1 and, to a lesser extent, ST13, responded strongly to dnHSF1 (Table 2).

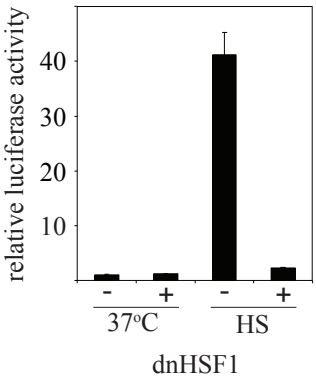


Figure 2 The effects of dnHSF on basal and heat shock-induced activity of an Hsp70 promoter. HEK293 cells carrying a stably integrated copy of the HSF379 (dnHSF1) were cultured in the absence (-) or presence (+) of doxycycline. Cells were transfected with a mixture of the Drosophila melanogaster Hsp70-luciferase reporter (pHL) and the Renilla Luciferase control plasmid pCMV-RL. At 48 hrs after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for 6 h and harvested. Hsp70 promoter activities were determined by dividing firefly luciferase values by the corresponding renilla luciferase (experiments using the HSF448 line) or β-galactosidase (experiments using the dnHSF1 line) values to correct for varying transfection efficiencies. The relative luciferase activity in cells cultured at 37°C in absence of the various HSF1 mutants was set at 1. The results are the average of three independent transfections (standard deviations are indicated by error bars).

To confirm the effect of HSF1 on the promoter activity of some of the genes downregulated by dnHSF1, we isolated the promoters and compared their activities in HEK-dnHSF1 cells and HEK-cDNA5 cells. The promoters of the STIP1, ST13, DNAJA1, DNAJB1 (see Table 2), and PMVK (selected because it is the strongest downregulated non-chaperone gene, Table 3) genes had significantly reduced activities in HEK-dnHSF1 cells compared with control cells, whereas the promoters of the unfolded protein response target genes CHOP and BiP, two genes with similar expression levels in HEK-dnHSF1 and control cells, were not or only slightly affected by dnHSF (Fig. 4). Note that these promoter activities were measured in unstressed cells, explaining why the activity of the promoter of the canonical heat stress inducible HSPA1A (Hsp70) gene is only inhibited by about 50%; note also that the activities of isolated promoter regions do not necessarily reflect the activity of the endogenous promoter which could also be controlled by chromatin structure and/or elements lacking from the isolated promoter region. The HSPB1 gene for example has been reported to have heat shock elements in its first intron as well [52].

Lack of heat shock proteins could cause stress in the cells, which in turn could activate a non-HSF dependent stress response (see also [53]). To determine whether exogenous expression of dnHSF1 caused stress we determined whether expression of dnHSF1 is associated with an increased

level of phosphorylated eIF2α. Activation of eIF2α kinases is a common response to a variety of stresses (for review, see [54]). As shown in Figure 5, the basal level of eIF2α phosphorylation is not increased by the expression of dnHSF1. In addition, the decay of eIF2α phosphorylation after a heat shock is not notably affected by expression of dnHSF1 (Fig. 5). This is in accordance with previous reports showing that cells lacking HSF1 are not impaired in their ability to recover from heat stress but do not built up thermostability after a heat stress [26, 55].

Table 2 Effect of exogenous expression of dnHSF1 on the transcript levels of the members of the families of heat shock proteins and their co-chaperones.

Gene name	Acc. Nr.	dnHSF1/Ctrl		Alternative name
		ave	sd	
HSPH family				
HSPH1	NM_006644	0.78	0.08	heat shock 105kDa/110kDa protein 1
HSPH2	NM_002154	0.66	0.04	heat shock 70kDa protein 4
HSPH3	NM_014278	0.61	0.21	heat shock 70kDa protein 4-like
HSPH4	NM_006389	1.19	0.33	hypoxia up-regulated 1
HSPA family				
HSPA1A/B1	NM_005345	0.93	0.18	hsp72
HSPA1L	NM_005527	not on array[2]	not on array[2]	heat shock 70kDa protein 1-like
HSPA2	NM_021979	1.22	0.20	
HSPA5	NM_005347	1.18	0.35	GRP78, BiP
HSPA6	NM_002155	0.46[3]	0.10	HSP70B'
HSPA8	NM_153201	0.87	0.08	HSC70
HSPA9	NM_004134	0.90	0.08	mortalin-2 (mitochondrial protein)
HSPA12A	NM_025015	1.10	0.20	KIAA0417
HSPA12B	NM_052970	nd[4]	nd[4]	
HSPA13	NM_006948	0.57	0.53	STCH
HSPA14	NM_016299	0.85	0.13	
HSP90 family				
HSP90AA1	NM_005348	0.38	0.06	Hsp90α
HSP90AB1	NM_007355	0.89	0.06	Hsp90β
HSP90B1	NM_003299	1.16	0.36	Grp94
TRAP1	NM_016292	1.06	0.05	TNF receptor-associated protein 1 (mitochondrial Hsp90)
DNAJ (Hsp40) family				
DNAJA1	NM_001539	0.64	0.10	HDJ2
DNAJA2	NM_005880	1.30	0.42	
DNAJA3	NM_005147	1.00	0.13	
DNAJA4	NM_018602	nd[4]	nd[4]	
DNAJB1	NM_006145	0.25	0.05	hsp40
DNAJB2	NM_006736	0.60	0.07	HSJ1
DNAJB3	NM_001001394	nd[4]	nd[4]	
DNAJB4	NM_007034	0.94	0.09	
DNAJB5	NM_012266	0.97	0.10	
DNAJB6	NM_005494	0.93	0.12	
DNAJB7	NM_145174	nd[4]	nd[4]	
DNAJB8	NM_153330	nd[4]	nd[4]	
DNAJB9	NM_012328	1.22	0.17	
DNAJB11	NM_016306	1.15	0.39	
DNAJB12	NM_001002762	1.04	0.11	
DNAJB13	NM_153614	nd[4]	nd[4]	

DNAJB14	NM_024920	0.87	0.04	
DNAJC1	NM_022365	1.17	0.23	
DNAJC2	NM_014377	0.89	0.06	zuotin related factor 1 (ZRF1)
DNAJC3	NM_006260	0.97	0.18	
DNAJC4	NM_005528	0.99[4]	0.10	
DNAJC5	NM_025219	nd[4]	nd[4]	cysteine string protein (CSP)
DNAJC5B	NM_033105	nd[4]	nd[4]	cysteine string protein beta (CSP-beta)
DNAJC5G	NM_173650	1.05[3]	0.07	
DNAJC6	NM_014787	0.87[3]	0.18	
DNAJC7	NM_003315	1.01	0.15	
DNAJC8	NM_014280	0.92	0.06	
DNAJC9	NM_015190	0.98	0.10	
DNAJC10	NM_018981	1.11	0.24	
DNAJC11	NM_018198	1.12	0.14	
DNAJC12	NM_021800	1.05	0.19	
DNAJC13	NM_015268	0.99	0.17	
DNAJC14	NM_032364	1.08	0.16	
DNAJC15	NM_013238	0.68	0.24	
DNAJC16	NM_015291	1.09	0.10	
DNAJC17	NM_018163	1.04	0.11	
DNAJC18	NM_152686	0.99	0.14	
DNAJC19	NM_145261	0.99	0.13	
DNAJC20	NM_172002	1.07[3]	0.12	J-type co-chaperone HSC20 (RP3-366L4.2)
DNAJC21	NM_194283	0.79	0.18	DnaJA5
DNAJC22	NM_024902	1.06	0.08	hypothetical protein FLJ13236
DNAJC23	NM_007214	0.98	0.08	SEC63
DNAJC24	NM_181706	0.87	0.11	ZCSL3
DNAJC25	NM_001015882	0.99	0.08	DnaJ-like protein (bA16L21.2.1)
DNAJC26	NM_005255	1.07	0.19	cyclin G associated kinase (GAK)
DNAJC27	NM_016544	0.98	0.10	Ras-associated protein Rap1 (RBJ)
DNAJC28	NM_017833	0.73[3]	0.18	C21orf55
DNAJC29	NM_014363	0.93	0.04	sacsin
DNAJC30	NM_032317	1.04	0.06	WBSCR18
HSPB (sHsp) family				
HSPB1	NM_001540	0.29	0.13	Hsp27
HSPB2	NM_001541	nd[4]	nd[4]	MKBP
HSPB3	NM_006308	nd[4]	nd[4]	
HSPB4	NM_000394	nd[4]	nd[4]	αA-crystallin (CRYAA)
HSPB5	NM_001885	0.99[3]	0.18	αB-crystallin (CRYAB)
HSPB6	NM_144617	1.04[3]	0.25	Hsp20
HSPB7	NM_014424	nd4	nd[4]	cvHsp
HSPB8	NM_014365	nd4	nd[4]	HSP22
HSPB9	NM_033194	0.68	0.20	
HSPB10	NM_024410	nd[4]	nd[4]	ODF1
Others				
HSPD1	NM_002156	0.88	0.17	Hsp60, chaperonin
HSPE1	NM_002157	0.73	0.08	Hsp10, chaperonin 10
SERPINH1	NM_001235	0.55	0.08	Hsp47
CCT3	NM_005998	0.67	0.17	TCP1, subunit 3 (gamma)
Co-chaperones				
AHSA1	NM_012111	0.63	0.07	AHA1 homolog 1
AHSA2	NM_152392	0.51	0.04	AHA1 homolog 2
BAG1	NM_004323	1.03	0.16	
BAG2	NM_004282	1.10	0.13	
BAG3	NM_004281	1.31	0.18	
BAG4	NM_004874	1.28[3]	0.43	
BAG5	NM_001015049	0.99	0.17	
PTGES3	NM_006601	0.88	0.14	p23
ST13	NM_003932	0.63	0.08	HIP
STIP1	NM_006819	0.53	0.06	HOP
STUB1	NM_005861	0.97	0.06	CHIP

AIP	NM_003977	0.94	0.21	
CDC37	NM_007065	nd[4]	nd[4]	
FKBP4	NM_002014	1.00	0.23	
FKBP5	NM_004117	0.98	0.07	
PPID	NM_005038	0.97	0.08	cyclophilin D
PPP5C	NM_006247	1.21	0.30	
SGTA	NM_003021	1.14	0.24	
TOMM70A	NM_014820	1.11	0.25	
TTC4	NM_004623	1.00	0.04	
UNC45A	NM_018671	0.99	0.07	

1 the array oligonucleotides do not discriminate between the transcripts of these two genes.
2 none of the oligonucleotides on the array hybridize with the transcript of this gene
3 the hybridization signal was significant but below 100
4 the hybridization signal was not significant

Glucocorticoid signalling is impaired by dnHSF1 and can be rescued by individual co-chaperones.

Expression of dnHSF1 depletes the cell of a number of chaperones and is predicted to decrease the activity of both the Hsp70 and the Hsp90 folding machine. Both are known to be important for maturation and function of steroid hormone receptors (reviewed in [42], [56]) and we thus examined whether expression of dnHSF1 resulted in impaired glucocorticoid hormone signalling. A synthetic glucocorticoid-responsive element (GRE) was linked to a luciferase reporter and used to monitor the response of HEK-dnHSF1 and HEK-cDNA5 cells to increasing concentrations of dexamethasone. Dexamethasone inducibility of the GRE was at least 50% inhibited in HEK-dnHSF1 cells compared with HEK-cDNA5 cells (Fig. 6). At 10^{-6} M dexamethasone, activity of the GRE was induced by 9-fold in HEK-cDNA5 cells and only by 4-fold in HEK-dnHSF1 cells, and at the highest concentration of dexamethasone the inducibility in HEK-cDNA5 cells was even 13-fold compared with only 5-fold in HEK-dnHSF1 cells.

Table 3 Non-chaperone encoding genes downregulated by dnHSF1.

Gene name	Acc. Nr.	dnHSF1/Ctrl		Description
		ave	sd	
PMVK	NM_006556	0.21	0.07	phosphomevalonate kinase
KLRG1	NM_005810	0.35	0.14	killer cell lectin-like receptor subfamily G, member 1
CDKL3	NM_016508	0.39	0.17	cyclin-dependent kinase-like 3
KA21	NM_152349	0.41	0.32	truncated type I keratin KA21
ZNF473	NM_015428	0.48	0.07	zinc finger protein 473
MLH1	NM_000249	0.50	0.17	mutL homolog 1

If the impaired dexamethasone inducibility in the presence of dnHSF1 is due to a reduction in the expression levels of one or more (co-)chaperone genes, then it should be possible to rescue the glucocorticoid inducibility of the GRE in HEK-dnHSF1 cells by exogenous expression of those (co-)chaperones. We therefore tested the effect of exogeneous expression of different proteins

on the glucocorticoid response of the pGRE-Luc reporter in HEK-dnHSF1 cells (Figs. 7 and 8).

Figure 3 Left panel. The decay of heat shock protein levels during expression of dnHSF1.
HEK-HSF379 cells were treated with doxycyclin for the time indicated and harvested. Right panel. The level of heat shock proteins in MEF wild type cells (+/+) and MEF cells lacking HSF1 (-/-) either before (-HS) or after heat shock and recovery (+HS). Cell lysates were subjected to SDS-PAGE and western blot analysis using the indicated antibodies.

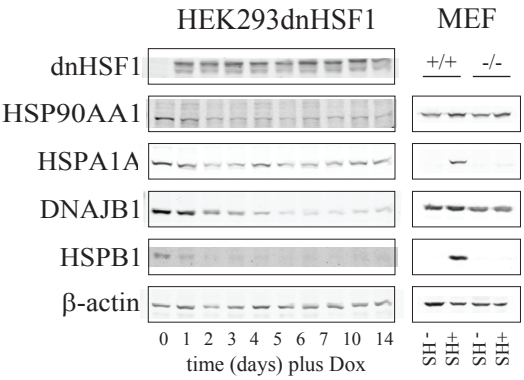
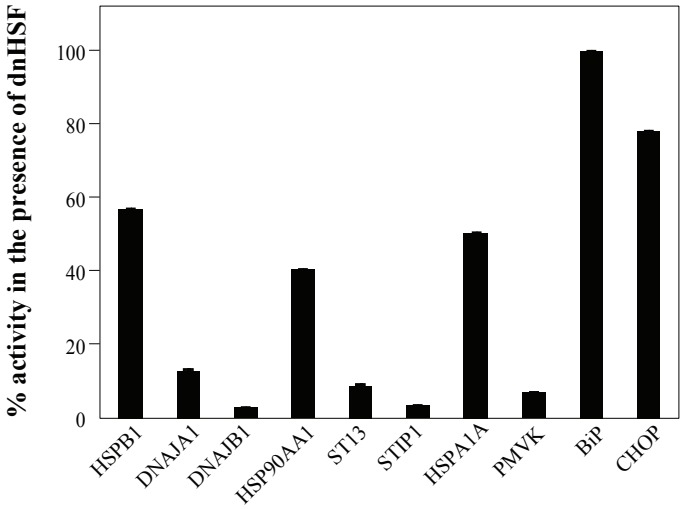


Figure 4 Inhibition of promoter activity by dnHSF1. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with the indicated promoter reporter constructs (see also Materials and Methods) and a β-actin-β-gal reporter. At 48 hr after transfection, cells were harvested and assayed for reporter gene activities. Promoter activities were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies. The bars correspond to the % activity of the promoter in the HEK-HSF379 cells compared with the control HEK-cDNA5 cells. The results are the average of three independent transfections (standard deviations are indicated by error bars).



The chaperone of which the expression is most effected by dnHSF1 is HSPB1. Although HSPB1 is not directly involved in the maturation of the glucocorticoid receptor, its lack may cause overloading of part of the folding network of the cell. However, exogenous expression of HSPB1 or of another sHsp, HSPB8, had no effect (Fig. 7). The level of Hsp90 is also affected by dnHSF1 but is apparently not limiting in the glucocorticoid response, as exogenous expression of Hsp90 was even inhibitory (Fig. 7). PTGES3 (p23) inhibited the GRE response in HEK-cDNA5 cells (Table 4) as previously reported [57, 58] but increased it slightly in HEK-dnHSF1cells. STIP1 (Hop), which is a co-chaperone of Hsp90 as well as of Hsp70 had no effect, either in HEK-cDNA5 (Table 4) or in HEK-dnHSF cells (Fig. 7). In contrast, ST13 (Hip), an Hsp70 co-chaperone, did restore dexamethasone inducibility to almost the wild type level in HEK-dnHSF cells. Even more effective was exogenous expression of the Hsp70 co-chaperones DNAJA1 (HDJ2) or DNAJB1 (Hsp40): this resulted in even higher dexamethasone inducibility in HEK-dnHSF1 cells compared with HEK-cDNA5 cells (Fig. 7). The rescue effect of DNAJA1 and DNAJB1 was not a general property of Hsp40 family members, since two other members of the DNAJB family, DNAJB6 and DNAJB8, did not show any rescue activity (Fig. 7). Expression of Hsp70 (HSPA1A) itself had no effect (Fig. 7; note that neither overexpression of DNAJ proteins nor overexpression of HSPA1A in HEK-cDNA5 cells affected the GRE response, see Table 4). These data show that it is the primary folding of the glucocorticoid receptor by the Hsp70 machinery that is most affected in HEK-dnHSF1 cells. As predicted by the wild-type level of DNAJB1 in *hsf1-/hsf1-* MEFs, these cells showed a wild-type glucocorticoid response (data not shown).

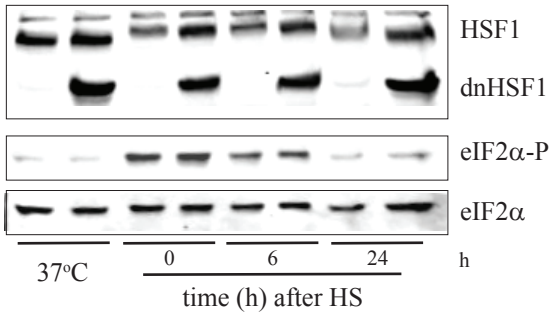


Figure 5 The effect of exogenous expression of dnHSF1 on eIF2α phosphorylation. HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin for 48 h. Cells were then exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and western blot analysis using the indicated antibodies.

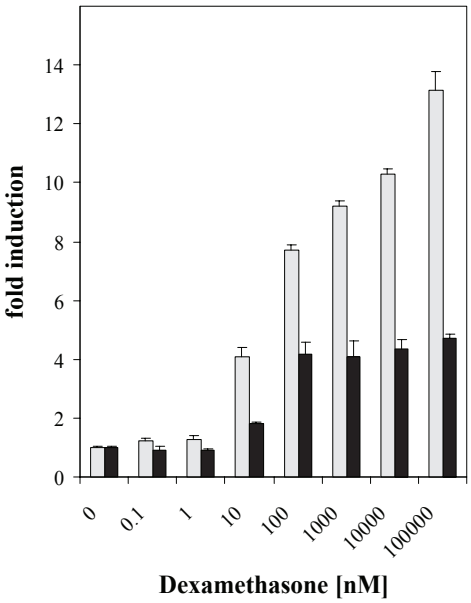


Figure 6 Exogenous expression of dnHSF1 reduces the glucocorticoid response. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with a glucocorticoid-responsive luciferase reporter (pGRE-Luc) and a β-actin-β-gal reporter. At 24 hr after transfection, cells were either left untreated or exposed to the indicated concentrations of dexamethasone. At 48 h after transfection, cells were harvested and assayed for reporter gene activities. Promoter activities were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies. The bars correspond to the activity of the glucocorticoid-responsive promoter in the presence of dexamethasone compared to the activity in untreated cells, which was set at 100%. Gray bars show the results for control HEK-cDNA5 cells; black bars those for HEK-HSF379 cells. The results are the average of three independent transfections (standard deviations are indicated by error bars).

Discussion

Comparison of the transcriptome of embryonic fibroblasts from HSF1 null mice with that of wild type cells identified 49 genes (19 related to immune response) that were not upregulated by a heat shock in wild type cells but nevertheless were expressed at reduced levels in HSF1 null fibroblasts [27]. When HSF1 was depleted by RNA interference in HeLa cells, the expression level of 378 genes changed significantly in the absence of stress [59]. The main effect, surprisingly, was an increase in expression, for 80% of the affected genes, the transcript level increased. In contrast, we found no significant increase in expression in response to dnHSF1; dnHSF1 reduced the expression level of only 10 genes more than two-fold, with a lesser effect on a number of chaperone encoding genes (Tables 2 and 3). The difference between the effect of depleting HSF1 in MEFs and HeLa cells is very likely to be caused by the far greater dependence of transformed cells on HSF1 [60]. HEK293 are less dependent on HSF1 than HeLa cells [60], but more so than MEFs. The response to blocking HSF1 in HEK293 cells might then be expected to be intermediate in the effect on the transcriptome but it is not. Clearly there is a difference between depleting HSF1 and expressing a dominant negative mutant. In part this difference may be due to a secondary effect: depletion of HSF1 would free the chaperones which are usually complexed with HSF1 while dnHSF1 might capture more chaperones. More importantly is probably the activity of HSF1 as a repressor of transcription.

Recently, it has been shown that HSF1 binds to MTA1, a co-repressor, to form a complex repressing estrogen-dependent transcription in breast carcinoma cells [30]. Similarly, HSF1 has been reported to interact with C/EBPβ, an interaction which represses transcriptional activation [61]. The loss of HSF1 would release repression; expression of dnHSF1 could maintain it. Expression of dnHSF1 is an efficient way of reducing the chaperoning capacity of the cell, as evidenced by the loss of the basal glucocorticoid response. Since the expression of so many genes playing roles at several stages of glucocorticoid receptor processing was suppressed in HEK-dnHSF1 cells, we did not expect that over-expression of individual proteins would rescue the glucocorticoid response. Nonetheless, the individual co-chaperones DNAJA1, DNAJB1 and ST13/Hip were able to rescue the dnHSF-mediated inhibition of the glucocorticoid response fully; PTGES3/p23 had some effect, whereas over-expression of Hsp90, or STIP1/Hop had no effect. Hsp90 was even inhibitory (Fig. 7). Both DNAJ and ST13/Hop are co-chaperones of Hsp70 and function in the primary folding of the glucocorticoid receptor, but at different levels: DNAJ activates the ATPase of Hsp70, whereas ST13/Hip stabilizes the Hsp70-ADP state (reviewed by [42]). Apparently over-expression of DNAJA1 or DNAJB1 can compensate for a shortage of ST13/Hip and vice versa, as exogenous expression of either protein restores glucocorticoid sensitivity. Together these data show that the limiting node of chaperoning network in dnHSF1 expressing cells is the Hsp70 folding machine, which is in turn is limited not by the level of Hsp70 itself, but rather by its co-chaperones. In vitro folding studies of the glucocorticoid receptor have shown that DNAJB1 is required in catalytic amounts [62]. Our data also show that a lack of DNAJB1 can be compensated for by overexpression of DNAJA1. Functional redundancy between DNAJB1 and another co-chaperone is also implied by the lack of a phenotype of the DNAJB1 knock-out mouse, which has only a minor deficiency in acquired thermotolerance [63]. In the case of the progesterone receptor it has been shown that either DNAJA1 or DNAJB1 can assist in folding but by distinct mechanisms. DNAJA1 bound tightly to the progesterone receptor while DNAJB1 did so only transiently [64].

Heat stress or expression of a dominant positive HSF1 mutant potentiates the glucocorticoid response [43,44] suggesting that the chaperone network is limiting for this response in normal cells. The chaperone network is also limiting for luciferase refolding as this can be boosted by overexpressing Hsp70, an effect which can be blocked by expressing a dominant negative DNAJB1 mutant [65]. In contrast, exogenous expression of single (co-) chaperones did not enhance the sensitivity of HEK-cDNA cells to dexamethasone, indicating that, unlike luciferase refolding, it is either a combination of chaperones and co-chaperones that is limiting or that other proteins are involved. In addition, exogenous expression of a dominant negative DNAJB1 mutant did not block the dexamethasone response significantly (data not shown).

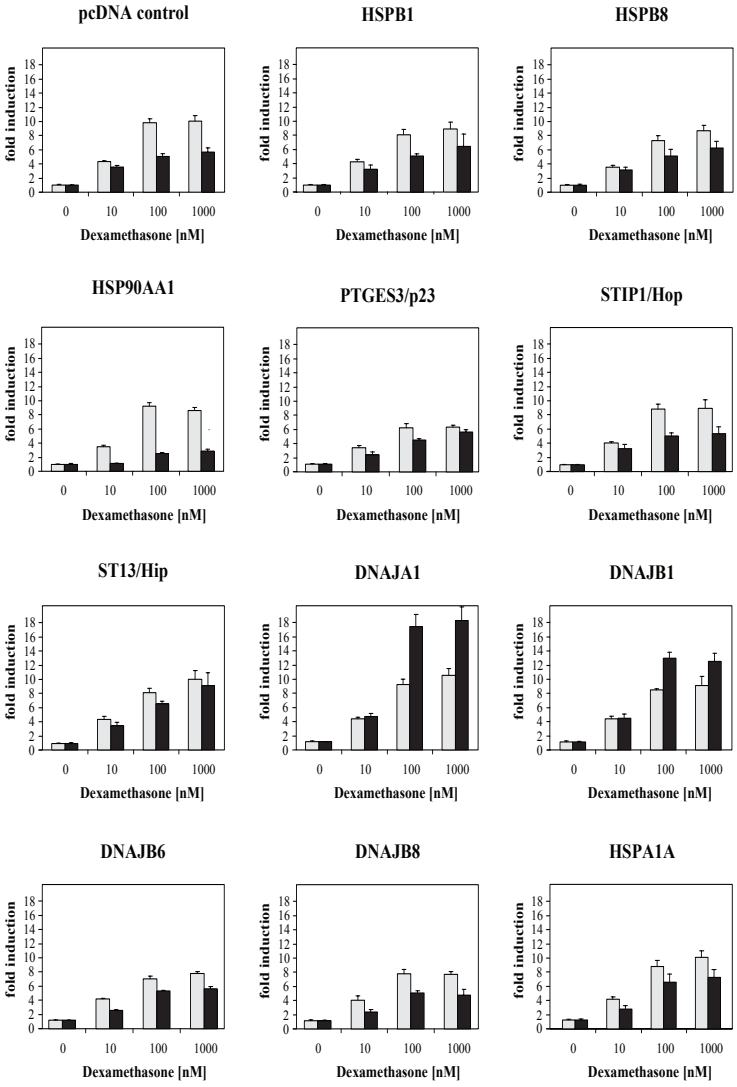


Figure 7 Effect of over-expression of (co)chaperones on glucocorticoid signaling in HEK-cDNA5 and HEK-dnHSF1 cells. Control HEK-cDNA5 cells (light gray bars) and HEK-HSF379 cells (black bars) were treated with doxycyclin. After 3 days, cells were transfected with a mixture (4:1:5) of glucocorticoid-responsive luciferase reporter (pGRE-Luc), a βactin-βgal reporter, and the expression construct indicated in the Figure. At 24 hr after transfection, cells were either left untreated or exposed to the indicated concentrations of dexamethasone. At 48 h after transfection, cells were harvested and assayed for reporter gene activities. Relative luciferase activities and -fold induction were determined as described in the legend to Figure 6. Standard deviations are indicated by the error bars.

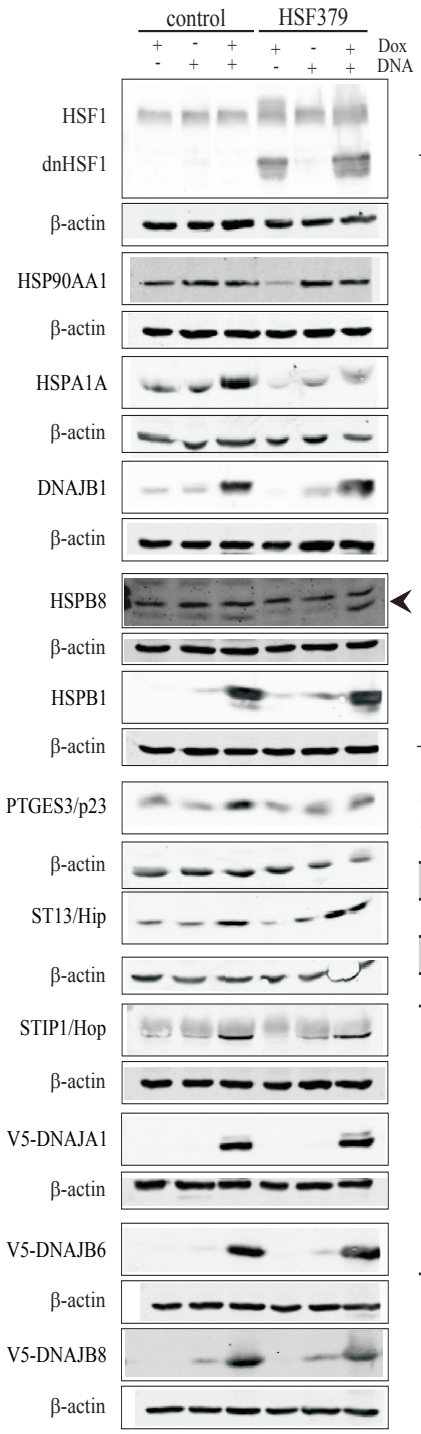


Figure 8 Levels of exogenous expression of (co)chaperones. Expression plasmids for the (co) chaperones indicated on the left were transfected into either HEK-cDNA cells (control) or HEK-HSF379 cells (+DNA) and expression was induced by adding doxycyclin (+Dox), except for HSP90AA1, of which expression is constitutive. Protein levels were determined by western blotting and staining with the corresponding antibody (see Materials and Methods). The arrowhead indicates HSPB8. Note that in the case of DNAJA1, DNAJB6 and DNAJB8 antibody to the V5-tag carried by the exogenous proteins was used; the endogenous protein is thus not detected. β-actin was used as a loading control.

Table 4 Relative effect of exogenous expression of (co)-chaperones on glucocorticoid signaling in HEK-cDNA5 cells.

Dexamethasone (nM)			
	10	100	1000
Gene name	*(co-)chaperones/control*		
	Ave + sd	Ave + sd	Ave + sd
HSPB1	1.0 + 0.1	0.8 + 0.1	0.9 + 0.2
HSPB8	0.8 + 0.1	0.7 + 0.1	0.8 + 0.1
HSP90AA1	1.1 + 0.3	0.9 + 0.2	0.9 + 0.3
PTGES3	0.8 + 0.1	0.7 + 0.1	0.6 + 0.1
STIP1	1.0 + 0.1	0.9 + 0.1	0.9 + 0.2
ST13	1.1 + 0.1	0.9 + 0.1	1.1 + 0.2
DNAJA1	1.0 + 0.1	1.0 + 0.1	1.1 + 0.1
DNAJB1	1.0 + 0.1	1.0 + 0.1	1.0 + 0.2
DNAJB6	0.9 + 0.1	0.8 + 0.1	0.8 + 0.1
DNAJB8	0.9 + 0.2	0.9 + 0.1	0.8 + 0.1
HSPA1A	0.8 + 0.2	0.8 + 0.2	1.0 + 0.4

Maintaining proteostasis during ageing is expected to prevent or at least ameliorate age-related protein folding and inflammatory disease [6, 41]. One possible approach is to prevent the decline in HSF1 activity either by targeting HSF1 directly or by targeting longevity related factors which control HSF1 activity such as SIRT1 [38]. One potential drawback of this approach is that HSF1 also increases the risk of cancer, also an often age-related disease [60]. An alternative is to maintain the capacity of the chaperoning network by boosting a single (co)chaperones. The results reported here show that DNAJA1 and DNAJB1 are promising targets. The finding that MEF cells do have wild-type levels of DNAJB1 in the absence of HSF1 shows that HSF1 can be bypassed in the transcriptional regulation of the DNAJB1 gene.

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Chapter 5

Protein refolding in peroxisomes is dependent on an HSF1 regulated function

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Abstract

Expression of a dominant negative HSF1 mutant (dnHSF1), which amongst other effects depletes cells of HSF1 regulated chaperones, blocked post heat shock refolding of luciferase targeted to the cytoplasm, nucleus or peroxisomes, while refolding of endoplasmic reticulum (ER) targeted luciferase was inhibited by about 50%. Luciferase refolding in the cytoplasm could be partially restored by expression of HSPA1A and fully by both HSPA1A and DNAJB1. For full refolding of ER luciferase HSPA1A expression sufficed. Neither nuclear nor peroxisomal refolding was rescued by HSPA1A. A stimulatory effect of DNAJB1 on post heat shock peroxisomal luciferase refolding by was seen in control cells, while refolding in the cytoplasm or nucleus in control cells was inhibited by DNAJB1 expression in the absence of added HSPA1A. HSPB1 also improved refolding of peroxisomal luciferase in control cells, but not in dnHSF1 expressing cells. HSP90, HSPA5, HSPA6 and phosphomevalonate kinase (PMVK) (of which the synthesis is also downregulated by dnHSF1) had no effect on peroxisomal refolding in either control or chaperone depleted cells. The chaperone requirement for post heat shock refolding of peroxisomal luciferase in control cells is thus unusual in that it can be augmented by DNAJB1 or HSPB1 but not by HSPA1A; in dnHSF1 expressing cells expression of none of the (co-)chaperones tested was effective and an as yet to be identified HSF1 regulated function is required.

Introduction

Proteins are made as linear chains but are active as intricately folded units often associated in larger assemblies. In dilute solution in vitro many proteins can fold without help, but in the in vivo high protein concentration environment proteins need help from molecular chaperones which prevent protein aggregation and provide a folding surface. Molecular chaperones also bind unfolded proteins and either help these to refold or target these for degradation by the proteasome or via autophagy [1]. Cells contain an unknown but probably large number of chaperones dedicated to the folding of a single protein or assembly of a single complex (see for example [2]). The three main cellular compartments, cytoplasm, mitochondria and ER, each have a compartment dedicated general chaperoning network. These networks are similar in that they all contain related chaperones and associated factors which aid in folding (Hsp90 and Hsp70 machines; [3, 4]), chaperones which deliver substrates to the folding machines (DNAJ/Hsp40 proteins) and chaperones which can store unfolded protein for later refolding or degradation (the small heat shock proteins) [5]. For example, HSPA5 (GRP78, BiP) is the ER specific Hsp70 paralog and GRP94 the ER Hsp90 paralog [6], while HSPA9 [7] and TRAP1 [8] are the mitochondrial Hsp70 and Hsp90 paralogs. The nuclear compartment does not have its own chaperoning network. Nuclear proteostasis mostly relies on transient passage of cytoplasmic chaperones [9, 10] and the nucleus is a poor folding environment [11, 12]. Peroxisome specific chaperones have been found in plant [13, 14] but not in animal cells. In some, but not all [15, 16], studies chaperones were detected in the peroxisome proteome [17, 18], but it cannot be excluded that these were trace contaminants as these were either cytoplasmic, ER or mitochondrial proteins. Peroxisomes are thought to bud off from the ER but their matrix enzymes are imported from the cytoplasm [19]. Protein import in the peroxisome is unusual in that fully folded proteins and even oligomeric protein complexes can be imported [20]. Peroxisomal matrix proteins could thus be folded in the cytoplasm and no chaperoning would then be required in the peroxisomal matrix. However, the high peroxisomal production of ROS, together with the discovery of several ROS-metabolizing enzymes in peroxisomes [21-24] suggests that peroxisomes have to cope with oxidative stress, which makes it peculiar that no chaperones are found within this organelle. In spite of the lack of classical chaperones, heat denatured peroxisomal luciferase is refolded as efficiently as it is in other cellular compartments [11], suggesting that peroxisomes can deal with unfolded proteins.

The chaperoning capacity of the various cellular compartments can be augmented by additional synthesis of chaperones when proteostasis in that compartment is sensed to fail. Mitochondria have an Unfolded Protein Response, also referred to as UPRmt [25], as does the ER (UPRER ; [26]). Unfolding proteins in the cytoplasm result in the activation of heat shock factor

1 (HSF1) and the increased transcription of the HSF1 target genes, amongst which those encoding cytoplasmic chaperones, the heat shock proteins. The result is a temporary increase in the chaperoning capacity allowing the cells to deal with unfolded proteins either by refolding or degradation. The higher level of chaperones also provides protection to a second proteotoxic insult. Heat shocking a cell thus provides it with thermotolerance, i.e. resistance to a second heat shock [27-30]. Such tolerance can be induced in all compartments, including the peroxisomes [11], even though the existence of a UPRper has not been demonstrated.

We have previously shown that even under physiological conditions and at normal growth temperatures (i.e. without stress) HSF1 regulates the level of cytoplasmic chaperones. Expression of a dominant negative mutant of HSF1 in HEK293 cells depletes cells of the classical HSF1 regulated chaperones such as HSP90AA1 (Hsp90), HSPA1A (Hsp70), DNAJB1 (Hsp40) and HSPB1 (Hsp27) [31]. By exogenous expression of single chaperones, it can then be tested which chaperone is limiting for the folding/activity of a particular chaperone client. Besides providing a fundamental insight in the substrate specificity and the critical nodes of the protein folding network of the cell, such information is of interest as HSF1 loses its activity during aging, making the aging cell prone to protein folding disease [32, 33]. Increasing expression of a (co-) chaperone would be a way of restoring proteostasis.

A common way to probe the chaperoning capacity of a cellular compartment is to measure the refolding of heat denatured luciferase targeted to that compartment [11]. In control cells luciferase can be equally well refolded in the cytoplasm, the ER and the peroxisome, while the nucleus is a poor refolding environment. In vivo refolding of luciferase, at least in the cytoplasm, requires the Hsp70 folding machine [34] and can be increased by exogenous expression of HSPA1A [11, 35], HSPA1A and DNAJB1 [35] and HSPB1 [36]. We show here that expression of dnHSF1 affects the luciferase refolding capacity not only in the cytoplasm or the nucleus, but also in the peroxisomes and the ER. We further show that only in the peroxisomes refolding cannot be restored by exogenous expression of HSPA1A and DNAJB1. Intriguingly, peroxisomal refolding seems to be sensitive to changes in expression of HSPB1, a cytoplasmic protein.

Materials and Methods

Western blot analysis

Cell pellets were homogenized in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4x sample buffer (200 mM Tris–HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. Protein samples were separated in 10% polyacrylamide gels and

transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1: 1,000 dilution, monoclonal HSP90 antibody (610418, BD Biosciences) at a 1:1,000 dilution, polyclonal HSPB1 antibody, obtained from Dr. A. Zantema, at a dilution of 1:400, monoclonal HSPA1A antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal HSPA5 antibody, kindly donated by Prof. Dr. Ineke Braakman, was used at a dilution of 1:1000, polyclonal DNAJB1 antibody (SPA-400; Stressgen) at a 1:10,000 dilution, polyclonal PMVK antibody, obtained from Dr. H.R. Waterham [37], was used at a 1:500 dilution, monoclonal V5 antibody (R96025; Invitrogen) was used at a 1:5,000 dilution, polyclonal γ-tubulin antibody (GTU-88; Abcam) at a 1:1,000 dilution, and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye® 800CW conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32210, respectively; LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

Cell culture

Flp-In T-REx-293 cells were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen). The stable cell lines HEK-dnHSF1 and HEK-cDNA5 have been described previously [31]. Flp-In T-REx-293 cells stably transfected with Per-superluc-eGFP selected with 0.5 mg/ml Geneticin (Invitrogen) were described previously as well [11]. These cells were in addition stably transfected with the doxycycline-inducible plasmids pcDNA5-dnHSF1 or pcDNA5-FRT/TO to yield HEK-dnHSF1-Per-superluc-eGFP and HEK-cDNA5-Per-superluc-eGFP. Cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. Cell lines stably transfected with Per-superluc-eGFP were also cultured with Geneticin (0.5 mg/ml).

Microscopy

HEK-dnHSF1-Per-superluc-eGFP cells (cultured in presence or absence of doxycycline for 10 days) were plated on poly-L-lysine (0.001%, Sigma-Aldrich) coated coverslips. 48 hours later, cells were fixed in 4% paraformaldehyde. All images were obtained by Fluorescence Microscopy using the DMRA fluorescence microscope with a Cohu CCD camera and QFluoro 1.2 software.

Recombinant DNA constructs

The C-terminal truncation mutant of dnHSF1 containing codons 1-379 from HSF1 as well as pcDNA5-HSPB1 and pcDNA5-HSP90AA1were described earlier [31]. The plasmids pcDNA5-V5-DNAJB1 and pcDNA5-V5-HSPA1A, pcDNA5-V5-HSPA6, Cyt-superluc-eGFP, ER-superluc-eGFP and Nuc-superluc-eGFP were described in [11, 38]. pcDNA5-PMVK was made by PCR amplifying the cDNA from HEK293 RNA using the primers PMVK up 5'-agctaagcttagtggccgcgtccat-3' and PMVK low 5'-cctcagaatctagacccccc-3' and cloning the PCR fragment (HindIII-XbaI(bl)) into pcDNA5-FRT/TO (HindIII-XhoI(bl)).

Transfections, and reporter gene assays

Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with 0.2 µg plasmid per well.

For analysis of the refolding capacity in different organelles, cells were transfected with a mixture of 10 ng luciferase reporter plasmid and 30 ng β-actin-β-galactosidase and 160 ng expression plasmids. To transfect equal amounts of expression plasmid, transfections with one chaperone coding expression plasmid were supplemented with an empty vector. In experiments using HEK- Per-superluc-eGFP 40 ng β-actin-β-galactosidase was used because no additional luciferase reporter was needed. Heat shock was performed at 45 °C for 30 min. Cells were harvested after a recovery period of 1, 2, or 3 h at 37 °C as indicated. Cycloheximide (0.2 mg/ml) was added prior to heat shock to block de novo synthesis of luciferase. Cells were lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 10 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus;Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 10 µl cell lysate was mixed with 50 µl luciferin solution and luminescence was again measured with the Lumat luminometer. Data shown are the mean ± S.D. of two or more independent experiments with 4 replicates each. The data points used to describe the refolding capacity of an organelle were obtained trough subtraction of the luciferase activity directly after heat shock. These values varied between 0.1% for nuclear luciferase and 15% for ER luciferase and are presented in S1-S3.

Results

Experimental design.

To measure the chaperoning capacity in the nucleus, cytoplasm or ER we used expression constructs for luciferase-eGFP targeted to these different compartments [11] transiently transfected into HEK293 cells with or without

expression of dominant negative HSF1. Transient transfection of an expression construct for luciferase-eGFP with a peroxisomal targeting sequence leads to mislocalization [11, 39]. To monitor refolding in peroxisomes we therefore used a cell line stably transfected with both the peroxisomal targeted luciferase-eGFP expression construct [11] and the tetracycline inducible dnHSF1 expression construct. In pilot experiments we found that expression of dnHSF1 needed to be induced for at least 48 h to see a decrease in the post heat shock refolding of cytoplasmic luciferase. Thus when cells expressing dnHSF1 were used, refolding was measured 96 h after induction of dnHSF1 expression. The fact that there is a delayed response to induction of dnHSF1 indicates that it is not the expression of dnHSF1 itself that leads to a reduced refolding capacity but that it is a secondary effect of the expression of dnHSF1, such as chaperone depletion. The amount of active luciferase remaining in cells harvested directly after heat shock varied between 1 and 10% of the pre-heat shock value depending on the cellular compartment and the chaperone complement (Fig. S1). In the data presented below only the rate of refolding under the various experimental conditions is presented.

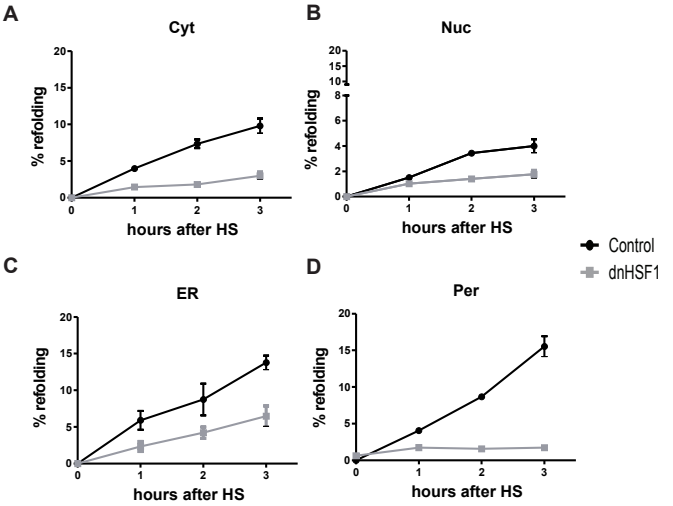

Figure 1 Effect dnHSF1 on refolding capacities of different organelles.
Relative luciferase activity of A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were harvested after heat shock at the times indicated and the luciferase activity was measured. The luciferase activity shown is relative to the activity in non heat shocked cells transfected and cultured in parallel. The results are the average of four independent transfections (standard deviations are indicated by error bars).

Effect of dnHSF1 expression on luciferase refolding in different cellular organelles.

Under our experimental conditions between 10% (cytoplasm) and 15% (ER, peroxisomes) of the luciferase was refolded 3 hrs post- heat shock in control cells. In the nucleus refolding was far less efficient and after 3 hrs only 4% of the luciferase was refolded (Fig. 1). As expected, dnHSF1 expression, which also causes chaperone depletion, severely inhibited post-heat shock luciferase refolding in the cytoplasm and nucleus. Unexpectedly, peroxiso-

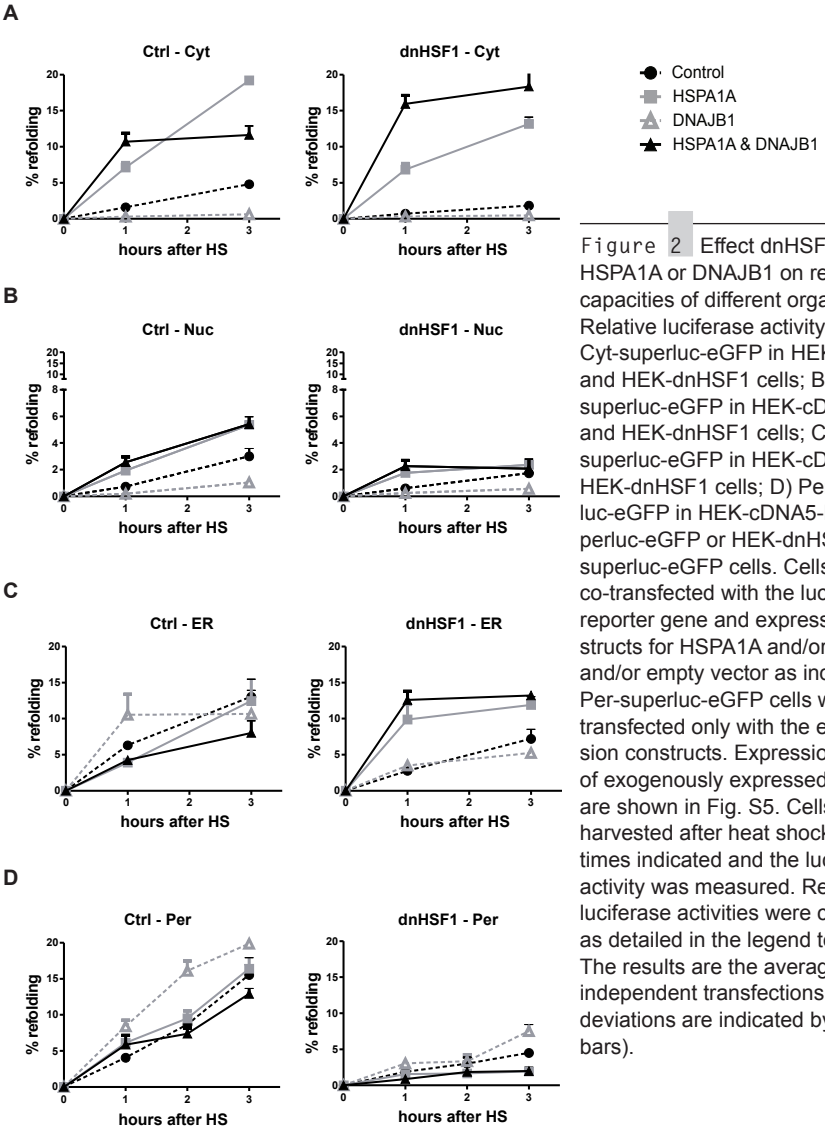

Figure 2 Effect dnHSF1 and HSPA1A or DNAJB1 on refolding capacities of different organelles. Relative luciferase activity of A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were co-transfected with the luciferase reporter gene and expression constructs for HSPA1A and/or DNAJB1 and/or empty vector as indicated. Per-superluc-eGFP cells were transfected only with the expression constructs. Expression levels of exogenously expressed proteins are shown in Fig. S5. Cells were harvested after heat shock at the times indicated and the luciferase activity was measured. Relative luciferase activities were calculated as detailed in the legend to Fig. 1. The results are the average of four independent transfections (standard deviations are indicated by error bars).

mal luciferase was also not refolded in dnHSF1 expressing cells. Refolding of ER targeted luciferase was less sensitive to dnHSF1 expression and was inhibited by about 50% (Fig. 1). Luciferase refolding is known to be mediated by the hsp70 folding machinery and refolding of cytoplasmic luciferase in dnHSF1 expressing cells could be completely restored by exogenous expression of both HSPA1A and DNAJB1. In control cells there was no synergistic effect between HSPA1A and DNAJB1 and expression of just DNAJB1even inhibited refolding (Fig. 2A), as previously reported [35]. The refold-

ing of nuclear luciferase in dnHSF1 expressing cells was not significantly improved by expression of either HSPA1A, DNAJB1 or both, while HSPA1A or HSPA1A and DNAJB1 did increase refolding of nuclear luciferase in control cells (Fig. 2B). DNAJB1 again inhibited. Exogenous expression of HSPA1A, DNAJB1 or both had little effect on the refolding of ER targeted luciferase in control cells but HSPA1A did restore ER luciferase refolding in dnHSF1 expressing cells. Exogenous expression of DNAJB1 had no effect. ER resident DNAJ proteins, which are not HSF1 regulated, might take the role of DNAJB1 in the ER. Unlike ER luciferase, refolding of peroxisomal luciferase in dnHSF1 expressing cells could not be restored by exogenous expression of HSPA1A. The peroxisomal compartment was the only compartment in which exogenous expression of DNAJB1 had a stimulatory effect on refolding: very slight in dnHSF1 expressing cells but significant in control cells (Fig. 2D). These data show that HSF1 regulated gene products do contribute significantly to the chaperoning capacity (as measured by post heat shock luciferase refolding) of all cellular compartments, including the ER and the peroxisomes. However, the critical nodes in the various compartments differ: HSPA1A restored the folding deficiency in dnHSF1 expressing cells in the cytosol and ER but did not so in nuclei or peroxisomes. The properties of the peroxisomal refolding network are unique in that it is the only compartment in which exogenous expression of DNAJB1 stimulated rather than inhibited refolding (in control cells).

The effect of chaperone depletion on refolding in ER and peroxisomes could be indirect: a deficit in cytoplasmic chaperoning capacity could have as a secondary effect the overloading of the ER and peroxisomal refolding machinery. We therefore tested if exogenous expression of HSPA5, an abundant ER chaperone, could compensate for depletion of the HSF1 regulated chaperones. Exogenous expression of HSPA5 had a significant effect on the thermostability of luciferase in all compartments, except the nucleus, as it about doubled the yield of luciferase activity directly after heat shock (data not shown). Curiously, this protective effect during heat shock did not correlate with an improvement in refolding after heat shock: exogenous expression of HSPA5 actually inhibited refolding in all compartments (data not shown).

Thermotolerance of peroxisomal refolding is dependent upon HSF1 regulated gene products.

Hageman et al. (2007) have shown that the refolding activity of the cytosol, nucleus, ER and peroxisomes increases in cells that have recovered from a heat shock. Such thermotolerance is due to the increased synthesis of chaperones. To show that the thermotolerance of the refolding in different cellular compartments requires HSF1 regulated gene products, we tested whether dnHSF1 expressing cells can acquire thermostability in the different cellular compartments. As shown in Fig. 3A, in cells that have been pre-heat shocked about 45% of the cytosolic luciferase was refolded within 1 hour

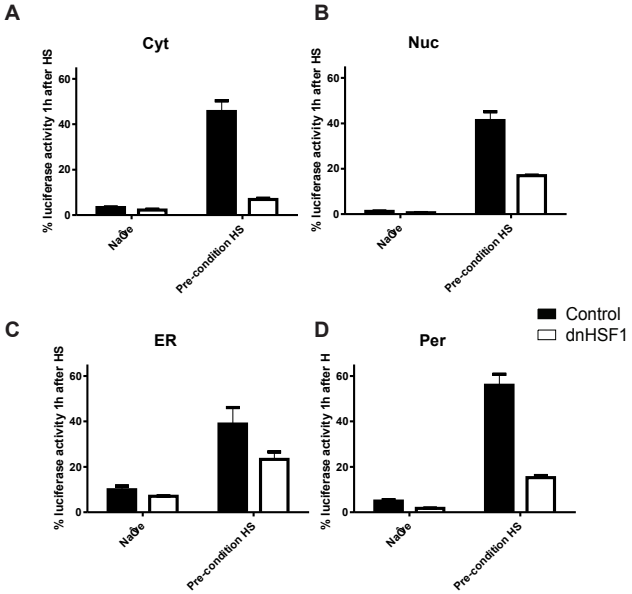

Figure 3 HSF1 dependent thermotolerance in different organelles. Thermotolerance in different cellular compartments. A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were either given a pre-heat shock at 45°C for 30 minutes or left untreated. 13 hours later, a second heat shock of 45°C for 30 minutes was applied. Cells were allowed to recover after heat shock for 1 hour. Relative luciferase activities were calculated as detailed in the legend to Fig. 1. The results are the average of four independent transfections (standard deviations are indicated by error bars).

post heat shock, while in naïve cells slightly more than 3% of the pre-heat shock luciferase activity was regained. Expression of dnHSF1 abolished the ability to induce thermotolerance, only 7% of the cytosolic luciferase was refolded in preconditioned dnHSF1 expressing cells (Fig. 3A). The acquired thermostability of nuclear compartment was less HSF1 dependent as 17% of the nuclear luciferase was refolded in preconditioned dnHSF1 expressing cells compared with 41% in normal preconditioned cells (Fig.3B). 23% of the luciferase targeted to the ER was refolded in preconditioned dnHSF1 expressing cells compared with 39% in normal preconditioned cells (Fig.3C), showing that the ER is less dependent on HSF1 for gaining thermostability. No chaperones have been detected in peroxisomes, yet luciferase can be refolded in peroxisomes [11, Figs. 1, 2]. Additionally, the refolding activity of the peroxisomes was shown to be increased in cells that have recovered from a heat shock. As shown in Fig. 3D, in cells that have been pre-heat shocked about 55% of the peroxisomal luciferase was refolded within 1 hour post heat shock, while in naïve cells had regained 5% of the pre-heat shock luciferase activity. Expression of dnHSF1 abolished the ability to induce thermotolerance, only 15% of the peroxisomal luciferase was refolded in preconditioned dnHSF1 expressing cells (Fig. 3D). These data show that it is the additional synthesis of HSF1 regulated gene products that is responsible for the improved refolding capacity of the peroxisomes in pre-heat shock cells and suggest that refolding of peroxisomal luciferase requires HSF1 regu-

lated chaperones. However, we were not able to rescue the inhibitory effect of dnHSF1 on gaining thermotolerance in peroxisomes by overexpressing chaperones encoded by HSF1 target genes (data not shown).

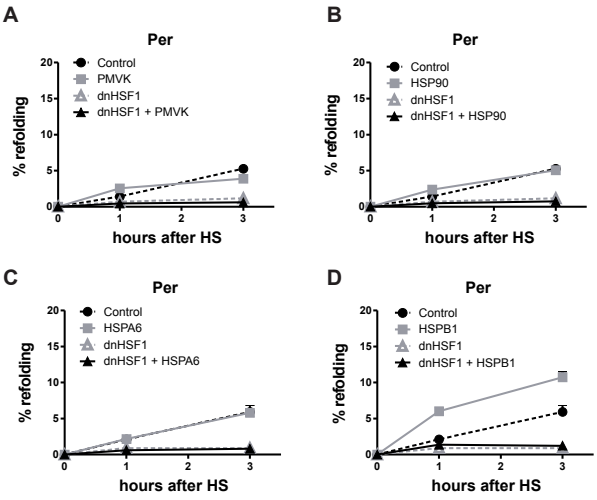

Figure 4 Effect of HSPA6, HSPB1, HSP90AA1 and PMVK on refolding capacities of peroxisomes.
Relative luciferase activity of Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were transfected with an expression constructs for PMVK (A), HSP90AA1 (B), HSPA6 (C) or HSPB1 (D). The expression levels of the exogenously expressed proteins are shown in Fig. S5. Cells were harvested after heat shock at the times indicated and the luciferase activity was measured. Relative luciferase activities were calculated as detailed in the legend to Fig. 1. The results are the average of four independent transfections (standard deviations are indicated by error bars).

HSPB1 promotes peroxisomal refolding.

As shown above, cells depend on HSF1 regulated genes for peroxisomal refolding in naïve cells as well as for the ability to develop thermotolerance of peroxisomal refolding. Yet, exogenous expression of the most likely candidates, the HSF1 dependent genes HSPA1A and DNAJB1, did not restore peroxisomal refolding (Fig. 2D). In the refolding assays, cycloheximide is added before the heat shock. The HSF1 regulated function must thus be one that is inhibited by dnHSF1 expression even in the non-stressed state. We have previously shown that of only 10 genes the transcript level is significantly (more than two-fold) lower when dnHSF1 is expressed in non-stressed cells [31]. Four of these encode (co)chaperone genes, HSP90AA1, HSPA6, DNAJB1 and HSPB1. Of the other six genes (PMVK, KLRG1, CDKL3, KA21, ZNF473, MLH1), only PMVK (phosphomevalonate kinase) looks like a possible candidate. The human PMVK sequence contains a putative peroxisomal targeting sequence [40] and was shown to be localized in peroxisomes [40, 41] although others have found PMVK to be mainly cytoplasmic [42]. A protein-protein interaction study detected multiple interactions of PMVK [43], which might indicate that PMVK could have a second role as a chaperone. We thus tested whether
exogenous expression of PMVK, HSP90AA1, HSPA6 or HSPB1 could rescue the decreased refolding capacity of peroxisomes in dnHSF1 expressing cells. Exogenous expression of none of these four proteins improved peroxi-

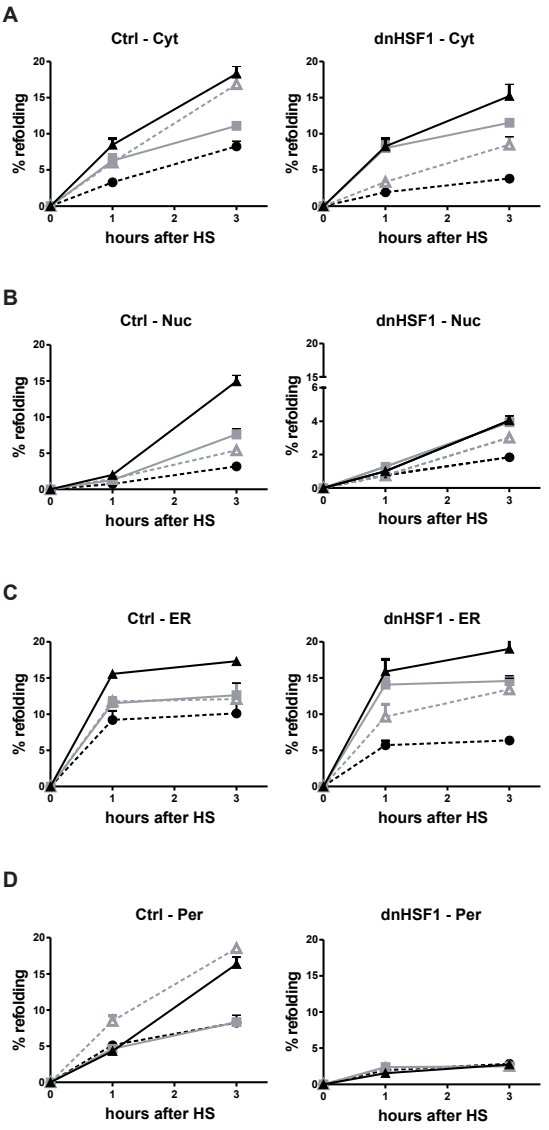

Figure 5 Effect HSPA1A and HSPB1 on refolding capacities of different organelles.
Relative luciferase activity of A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were co-transfected with the luciferase reporter gene and expression constructs for HSPA1A and/or HSPB1 and/or empty vector as indicated. Per-superluc-eGFP cells were transfected only with the expression constructs. Expression levels of exogenously expressed proteins are shown in Fig. S6. Cells were harvested after heat shock at the times indicated and the luciferase activity was measured. Relative luciferase activities were calculated as detailed in the legend to Fig. 1. The results are the average of four independent transfections (standard deviations are indicated by error bars).

somal refolding in dnHSF1 expressing cells (Fig. 4A-B; note that the extent of peroxisomal refolding shown here is somewhat lower than shown in other figures, presumably due to a slightly harsher heat shock). Intriguingly, in control cells HSPB1 did improve peroxisomal refolding (Fig. 4D).
HSPB1 cannot refold proteins but can maintain these in a refolding competent state and cooperate with the Hsp70 folding machine, which refolds the HSPB1 substrates. We thus tested whether the lack of an effect of HSPB1on peroxisomal refolding in dnHSF1 expressing cells was due to a

lack of HSPA1A. It was not: exogenous expression of HSPA1A had no effect (Fig. 5). In contrast, in other cellular compartment an effect of HSPB1 did require HSPA1A. The limiting node in the peroxisomal chaperone network thus differs from that in other compartments.
As exogenous expression of the traditional HSF1 target genes did not restore peroxisomal refolding in dnHSF1 expressing cells, we tested the effect of other chaperone family members as well. Exogenous expression of HSPH1, GRP94, HSPA8, HSP47(SERPINH1), DNAJA1, DNAJB2a, DNAJB2b, DNAJB6, DNAJB8, HSPB5 or HSPB8 had no effect on peroxisomal protein folding (data not shown).

Discussion

We show here that expression of dnHSF1 not only affects cytoplasmic and nuclear refolding but, unexpectedly, also refolding in the ER and the peroxisomes. The obvious explanation would be that expression of dnHSF1 depletes the cell of cytoplasmic, HSF1 regulated, chaperones. Peroxisomal matrix proteins are imported from the cytoplasm and thought to be folded by the cytoplasmic chaperones. How peroxisomes deal with unfolded proteins is not known. The ER has a retrograde export system for unfolded proteins (ERAD), which are then degraded in the cytoplasm. Peroxisomes are not known to have an export system but their import system shows a striking similarity to ERAD [44] with the crucial difference that ERAD exports while the peroxisomal system imports. Perhaps the peroxisomal import system is reversible and can also export when unfolded proteins accumulate in the peroxisomes. These proteins could then either be refolded and reimported or degraded. As we have shown here, peroxisomal refolding is fully dependent upon HSF1 activity and at first glance appears to equal to, and to be due to, cytoplasmic refolding. However, a closer examination shows important differences. Cytoplasmic refolding in control cells can be augmented by exogenous expression of HSPA1A and is thus limited by the Hsp70 machinery. Refolding of peroxisomal luciferase, in contrast, is limited by co-chaperones. In control cells it can be increased somewhat by exogenous expression of DNAJB1 which supplies substrate to Hsp70, and by HSPB1, which serves as a holding reservoir for Hsp70 refolding. Hsp70 activity itself is not limiting, as exogenous expression of HSPA1A has no effect on peroxisomal refolding. Peroxisomes thus appear to have Hsp70 activity in excess, while in the cytoplasm Hsp70 is limiting. A second important difference between cytoplasmic and peroxisomal refolding is that in cells expressing dn HSF1, refolding of peroxisomal luciferase cannot be restored by exogenous expression of any of the (co-)chaperones we tested, while expression of HSPA1A+DNAJB1 fully restored cytoplasmic luciferase refolding. This difference could be due to a spatial restriction of the refolding of peroxisomal proteins. In plants, a membrane bound DNAJ protein recruits cytoplasmic Hsp70 to the peroxisomal surface [45] and peroxisomal proteins could thus be refolded locally

on the cytosolic side of the peroxisomal membrane rather than dispersed in the cytoplasm. An alternative possibility to cytoplasmic refolding of peroxisomal proteins is that peroxisomes do import chaperones under conditions of proteotoxic stress. It has been shown that peroxisomes import unfolded BSA along with HSPA8 (Hsc70) [46]. A transient accumulation of chaperones in peroxisomes during proteotoxic stress could then ensure refolding. Whatever the mechanism of peroxisomal refolding is, it requires a crucial component of which the function is somehow dependent upon HSF1 regulated gene expression. What that component is, is unknown; none of the chaperones tested in our experiments restored peroxisomal refolding in dnHSF1 expressing cells. Expression of dnHSF1 did not impair import of peroxisomal targeted eGFP-luciferase: in cells cultured for 12 days with continuous expression of dnHSF1 peroxisomal eGFP-luciferase was not effected (Fig. S7).

The ER has its own set of chaperones. Except for SERPINH1 (Hsp47), the synthesis and level of the ER chaperones is not regulated by HSF1. Yet, we do find that expression of dnHSF1 also results in a 50% inhibition of refolding of ER targeted luciferase. We cannot rigorously exclude that some of the ER targeted luciferase is mislocalized in the cytoplasm. However, if that was the case we would expect that exogenous HSPA1A would improve ER luciferase refolding in control cells just as it improves cytoplasmic luciferase refolding. It did not. The most likely explanation is that somehow lack of cytoplasmic chaperones clogs up the ER refolding machinery. Cytoplasmic domains of integral membrane proteins are folded by cytoplasmic chaperones. In addition cytoplasmic Hsp70s are implicated in ERAD [47, 48]. In our hands a lack of cytoplasmic chaperones due to dnHSF1 expression could not be relieved by exogenous expression of HSPA5, the ER resident Hsp70. If anything, exogenous expression of HSPA5 inhibited post-heat shock refolding. Curiously, HSPA5 was effective in preventing denaturation during heat shock and HSPA5 was the only exogenously expressed chaperone that did not show a direct correlation between protection during heat shock and of post-heat shock refolding, suggesting that in this case these are distinct processes.

Previously we showed that the activity of the gluccocorticoid receptor, a client of both the Hsp70 and the Hsp90 machines, could be restored in dnHSF1 expressing cells by exogenous expression of DNAJB1 but not HSPA1A [31]. In contrast, exogenous expression of DNAJB1 did not restore refolding of luciferase, HSPA1A is required as well. Which node of the chaperoning network is critical thus depends on the substrate tested and the compartment in which the substrate is located. Increasing expression of (co-)chaperones to compensate for the loss of HSF1 regulated chaperones in aging cells thus needs to be tailored for specific substrates.

Acknowledgements

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Supplemental tables and figures

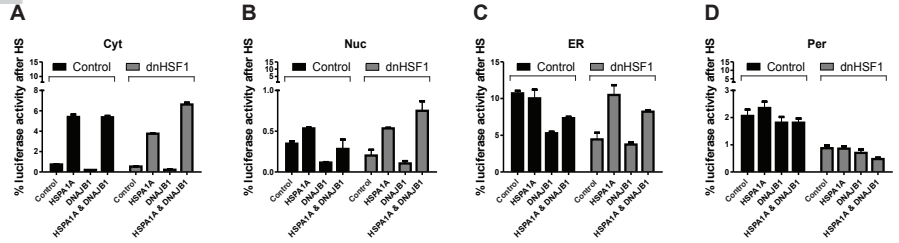

Figure S1 Effect dnHSF1 and HSPA1A or DNAJB1 expression on luciferase denaturation in different organelles Luciferase activity directly after HS of A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were co-transfected with the luciferase reporter gene and expression constructs for HSPA1A and/or DNAJB1 and/or empty vector as indicated. Per-superluc-eGFP cells were transfected only with the expression constructs. Cells were harvested directly after heat shock and the luciferase activity was measured. Luciferase activities were calculated relative to those in non heat shocked cells transfected and cultured in parallel. The results are the average of four independent transfections (standard deviations are indicated by error bars).

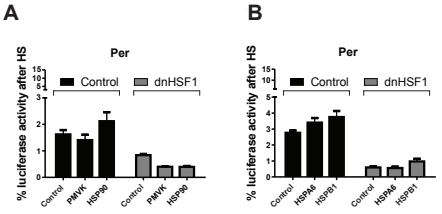

Figure S2 Effect of exogenous expression of PMVK, HSP90AA1, HSPA6 or HSPB1 on denaturation of peroxisomal luciferase. Luciferase activity directly after HS of Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were transfected with an expression constructs for HSPA6, HSPB1, HSP90AA1 or PMVK. Cells were harvested directly after heat shock and the luciferase activity was measured. Luciferase activities were calculated relative to those in non heat shocked cells transfected and cultured in parallel. The results are the average of four independent transfections (standard deviations are indicated by error bars).

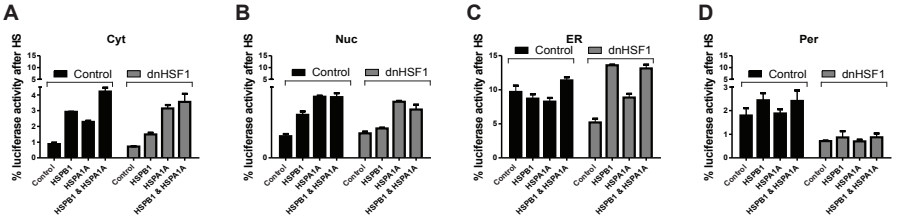

Figure S3 Effect of exogenous expression of HSPA1A and HSPB1 on luciferase denaturation in different organelles Luciferase activity directly after HS of A) Cyt-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; B) Nuc-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; C) ER-superluc-eGFP in HEK-cDNA5 and HEK-dnHSF1 cells; D) Per-superluc-eGFP in HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells. Cells were co-transfected with the luciferase reporter gene and expression constructs for HSPA1A and/or HSPB1 and/or empty vector as indicated. Per-superluc-eGFP cells were transfected only with the expression constructs. Cells were harvested directly after heat shock and the luciferase activity was measured. Luciferase activities were calculated relative to those in non heat shocked cells transfected and cultured in parallel. The results are the average of four independent transfections (standard deviations are indicated by error bars).

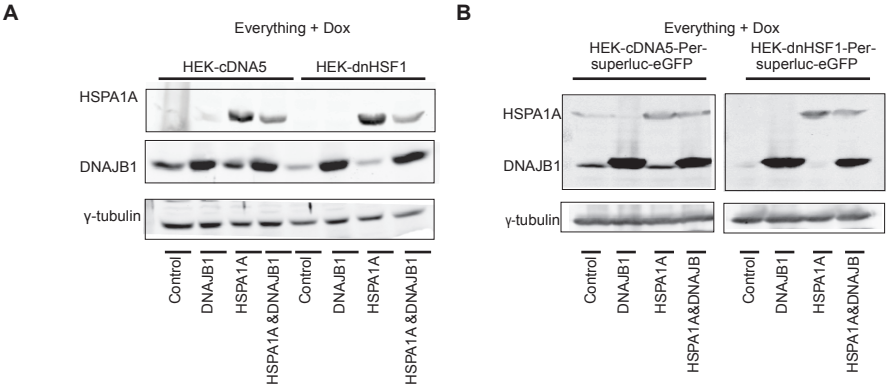

Figure S4 Exogenous expression of HSPA1A and DNAJB1.
Levels of exogenous expression of HSPA1A and DNAJB1. Expression plasmids for the (co-) chaperones indicated were transfected into either A) HEK-cDNA cells, HEK-HSF379, B) HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells and expression of dnHSF1 was induced by adding doxycyclin. Protein levels were determined by western blotting and staining with the corresponding antibody (see "Materials and methods"). γ-tubulin was used as a loading control.

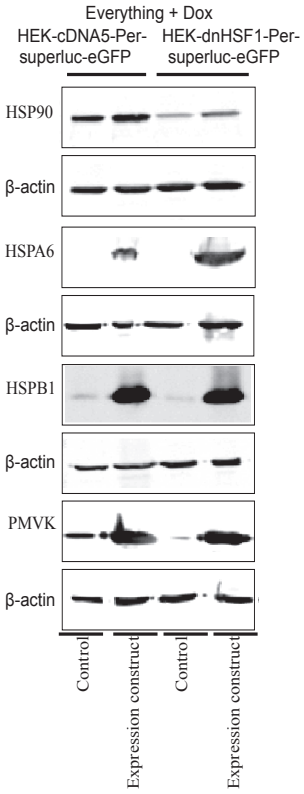

Figure S5 Exogenous expression of HSP90AA1, HSPA6, HSPB1 and PMVK.
Levels of exogenous expression of different (co-) chaperones. Expression plasmids for HSP90AA1, HSPA6, HSPB1 and PMVK were transfected into either HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells and expression was induced by adding doxycyclin. Except for HSP90AA1, of which expression is constitutive. Protein levels were determined by western blotting and staining with the corresponding antibody (see "Materials and methods"). Note that in the case of HSPA6 antibody to the V5-tag carried by the exogenous proteins was used; the endogenous protein is thus not detected. β-actin was used as a loading control.

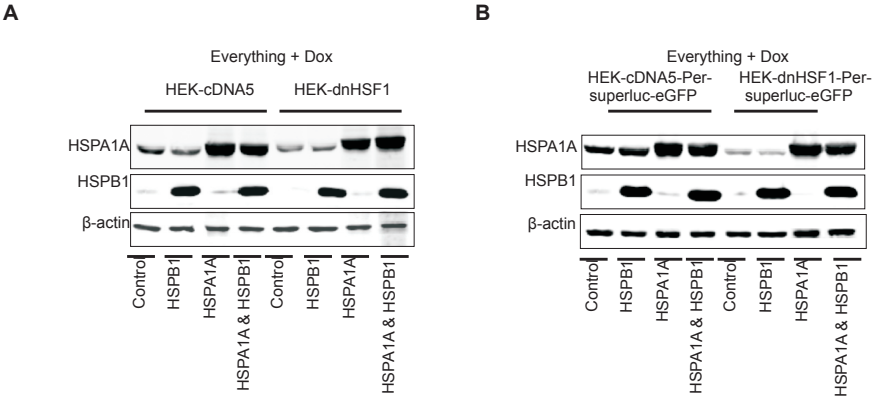

Figure S6 Exogenous expression of HSPA1A and HSPB1.
Levels of exogenous expression of HSPA1A and HSPB1. Expression plasmids for the (co-) chaperones indicated were transfected into either A) HEK-cDNA cells, HEK-HSF379, B) HEK-cDNA5-Per-superluc-eGFP or HEK-dnHSF1-Per-superluc-eGFP cells and expression was induced by adding doxycyclin. Protein levels were determined by western blotting and staining with the corresponding antibody (see ‘‘Materials and methods’’). β-actin was used as a loading control.

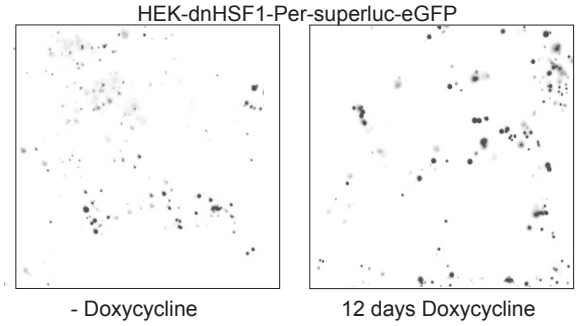

Figure S7 Effect of dnHSF1 expression on the localization of peroxisomal targeted luciferase.
Confocal micrographs showing eGFP of Per-superluc-eGFP in control cells (HEK-dnHSF1-Per-superluc-eGFP cultured in the absence of doxycycline) and in cells overexpressing dnHSF1 for 12 days.

Chapter 6

The 5'-untranslated region of the human PMVK gene contains an HSF1 binding site

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Abstract

Phosphomevalonate kinase (PMVK) catalyzes the ATP dependent phosphorylation of mevalonate 5-phosphate to produce mevalonate 5-diphosphate and ADP in the mevalonate pathway. We show here that the PMVK gene contains an HSE, the element binding heat shock factor 1 (HSF1; a transcription factor activated upon cytoplasmic proteotoxic stress), in the region encoding the 5' UTR. PMVK mRNA and protein levels were strongly downregulated not only when a dominant negative mutant of HSF1 (HSF379) was expressed, but also when an HSF1 mutant (HSF448), which has a weak dominant positive effect on traditional HSF1 responsive promoters, was expressed. The levels of PMVK mRNA were not affected by a heat shock and the activation of the PMVK promoter by sterol depletion was not HSF1 dependent. Whether the PMVK HSE plays a physiological role remains unclear.

Introduction

Cytoplasmic proteotoxic stress leads to activation of HSF1, which then mediates transcription of several genes amongst which genes encoding for chaperones known as the heat shock proteins [1-3]. HSF1 null mice lack the heat shock response and additionally show neuronal and developmental defects [4-6]. The latter phenotypes cannot be linked to the heat shock response directly, suggesting that HSF1 also regulates transcription of several genes under normal, non stress conditions. Direct evidence for the regulation of genes by HSF1 under non stress conditions was found in heregulin treated breast carcinoma cells. HSF1 repressed the estrogen-dependent transcription induced by heregulin [7]. It has also been discovered that Hsf1 deficient mice show altered circadian period lengths, suggesting that HSF1 is of functional importance for the circadian clock [8].
To identify HSF1 targets in the absence of stress we have used a dominant negative (dn)HSF1 mutant to inhibit HSF1 activity in non stressed cells. Besides some chaperone and co-chaperone genes also a number of genes not known as HSF1 targets were downregulated by dnHSF1 expression [9]. One of the latter is that encoding phosphomevalonate kinase (PMVK). PMVK is involved in the mevalonate pathway by catalyzing the ATP dependent phosphorylation of mevalonate 5-phosphate to produce mevalonate 5-diphosphate and ADP [10]. The mevalonate pathway is responsible for the synthesis of cholesterol from acetyl CoA and acetoacetyl CoA [10]. PMVK mRNA levels and the corresponding enzymatic activity are regulated in response to cellular sterol levels [11].The PMVK gene region has been shown to bind HSF1 in ChIP-on-chip experiments [12], albeit weakly. Furthermore, a protein-protein interaction study showed multiple interactions of PMVK [13] which might suggest that PMVK has a second role as a chaperone. We thus selected the PMVK gene for further study. To elucidate the possible role of HSF1 in regulating PMVK levels, we first mapped the HSF1 interaction site in the PMVK promoter region and found an HSE located in the PMVK gene region encoding the 5' UTR. However, we failed to find a function for this HSE: we could find no evidence for an involvement of HSF1 in regulation of the activity of the PVMK gene either in heat stressed or in sterol depleted cells.

Materials and Methods

Cell culture

Flp-In T-REx-293 cells were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-dnHSF1 (HSF379) , HEK-cDNA5, HEK-HSF448, HEK-HSF1 WT and HEK-HSF1-K80Q that carry a single copy of the tetracycline-inducible plasmids pcDNA5-dnHSF1, pcDNA5, pcDNA5-HSF448, pcDNA5-HSF1 WT and pcDNA5-HSF1-K80Q respectively. The cells were cultured at 37°C/5%

CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions.

RNA Isolation and Real Time Quantitative RT-PCR

Cells were washed twice with ice-cold PBS and after washing the cells, RNA was isolated with TRIzol (Invitrogen) according to manufacturer's recommendation. Isolated RNA samples were treated with RNase-free DNase I (Qiagen) for 15 minutes. cDNA was generated using the Reverse Transcription System from Promega following the manufacturer's instructions. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with Power SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in Table 1. Parallel PCR reactions were performed with 0.125 µg of the DNase treated RNA to test for DNA contamination. GAPDH mRNA was used as a control.

Transfections, and reporter gene assays

Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with 0.2 µg plasmid per well. For analysis of the PMVK promoter activity, cells were transfected with a mixture of 180 ng luciferase reporter plasmid and 20 ng β-actin-β-galactosidase except when the dpHSF1 was also expressed. In those experiments a mixture of 160 ng luciferase reporter plasmid, 20 ng expression plasmid or empty vector and 20 ng β-actin-β-galactosidase was used. At 24 hours after transfection, the expression of dnHSF1, HSF448, HSF1 WT, HSF1 K80Q or dpHSF1 was induced by adding 0.1 µg/ml doxycycline. At 48 hours after transfection, cells were heat shocked for 30 minutes at 45 °C. Cells were harvested after a recovery period at 37°C. For sterol depletion, cells were seeded in phenol red free medium supplemented with 10% charcoal stripped fetal bovine serum, GlutaMAX , 100 U/ml penicillin and 100 µg/ml streptomycin. After 24 hours, 1 or 10 µM lovastatin was added in phenol red free medium lacking serum and cells were cultured for an additional 24 hours.

Cells were lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 10 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM MgCl2, 1% Galacton-Plus;Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and lumines-

cence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 10 µl cell lysate was mixed with 50 µl luciferin solution and luminescence was again measured with the Lumat luminometer.

Recombinant DNA constructs

pcDNA5-dnHSF1, pcDNA5-HSF448 and pcDNA5-dpHSF1 have been described previously [14]. The pcDNA5-HSF1 silent mutation (HSF1 WT) and the pcDNA5-HSF1-K80Q mutant were made by performing site-directed mutagenesis on pcDNA5-HSF1 with respectively the HSF1 sil.mut and the HSF1-K80Q primers (listed in Table 1).

The pGL3-PMVK (-1188, +142) promoter clone was constructed by PCR on DNA isolated from human lymphocytes cells. PCR fragments were cloned into pGEM-T-Easy vector (Promega) and, after sequencing, the promoter sequence was cloned into the pGL3 basic vector. pGL3-PMVK (-816,+142) was made by digesting pGL3-PMVK (-1183,+147) with KpnI and AFLII (blunt ends). pGL3-PMVK (-620,+142) was made by digesting pGL3-PMVK (-1188,+142) with KpnI and PvuII (blunt ends). PMVK-5'UTR-SV40 was made by amplifying the 5' UTR region by PCR on pGL3-PMVK (-1188,+142) and inserting it in pGL3 promoter. PMVK-HSE-SV40 was made by annealing the corresponding primers and cloning the double-stranded oligo into the XhoI and NheI sites of pGL3-promoter. dmHsp70-HSE-SV40 was made by annealing the corresponding primers and cloning the double-stranded oligo into the XhoI and NheI sites of pGL3-promoter.

Chromatin Immunoprecipitation

HEK-dnHSF1 or HEK-cDNA5 cells were cultured for 48 h in the presence of doxycycline. Chromatin immunoprecipitation was performed as described in [15] except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES.KOH pH 7.6, 140 mM NaCl, 1mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor complete). Sonicated chromatin was centrifuged for 5 min at 4°C and then incubated overnight in incubation buffer (final concentration; 12 mM HEPES-KOH pH 7.6, 90 mM NaCl, 0.6 mM EDTA pH. 8.0, 0.09% SDS, 0.6% Triton X-100, 0.1% BSA) together with purified anti-HSF1 antibody (SPA-901; Stressgen; 1:1000) or anti-CEBPβ (sc-150; Santa Cruz; 1:1000) and protein A/G beads (Santa Cruz). Negative control without adding antibody was included. Beads were washed six times with different buffers at 4°C: twice with 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 150 mM NaCl, HEG (1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES-KOH pH 7.6), once with the same buffer but with 500 mM NaCl, once with 0.25 M LiCl, 0.5% NaDOC, 0.5% NP-40, HEG and twice with HEG. Precipitated chromatin was eluted with 400 µl of elution buffer (1% SDS, 0.1 M NaHCO3), incubated at 65°C for 4 h in the presence of 200 mM NaCl, phenol extracted and

precipitated with 20 µg of glycogen at -20°C overnight. ChIP experiments were analyzed by qPCR. Efficiency of ChIP was calculated as percentage of input. The primers used are listed in Table 1.

Western blot analysis

Cell pellets were homogenized in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4x sample buffer (200 mM Tris–HCl pH 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. Protein samples were separated in 10% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, monoclonal Hsp70 antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal PMVK antibody, obtained from Dr. H.R. Waterham [16], was used at a 1:500 dilution and mouse monoclonal β-actin antibody (AC-15, Sigma-Aldrich) was used at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye® 800CW conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32210, respectively; LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

Table 1 Oligonucleotides that were used.

	Oligo name	Oligo sequence (5' → 3')
Cloning	PMVK prom up	agctaagcttactcaggtaaaacaggagatgtg
	PMVK prom low	agctccatggccaaacagatatggggagaaaag
	PMVK 5'UTR up	agctcccgggaaggttctgggcggggctggactgttc
	PMVK 5'UTR low	agctccatggccaaacagatatggggagaaaag
	PMVK HSE up	ctagagggaaggttctgggcggggctggactgttctaagtgagttcgggtgc
	PMVK HSE low	tcgagcacccgaactcacttagaacagtccagccccgcccagaaccttccct
	dmHsp70 HSE up	ctagctctattctcgttgcttcgagagagcgcgcctcgaatgttcgcgaaaagagcg
	dmHsp70 HSE low	gatccgctcttttcgcgaacattcgaggcgcgctctctcgaagcaacgagaatagag
	HSF1 sil. mut. (HSF1 WT)	cagaaagtcgtcaacaagcttatccagttcctgatctcactg
	HSF1-K80Q	catgtatggcttccggcaagtggtccacatcgagc
ChIP	PMVK ChIP fwd	gtcagttaagcaggccaggc
	PMVK ChIP rev	ccaaacagatatggggagaaa
	DNAJB1 ChIP fwd	ggatgtcgcgtgtcgctgaa
	DNAJB1 ChIP rev	cgaccagtcccggactctata
QPCR	GAPDH_QPCR_fwd	gcagctgaaagaagcccaagt
	GAPDH_QPCR_rev	tgtcttccatgccaattgca
	HSPA1A_QPCR_fwd	ccgagaaggacgagtttgag
	HSPA1A_QPCR_rev	acaaaaacagcaatcttggaaagg
	HSP90_QPCR_fwd	gttggtcctgtgcggtcact

Results

Expression of dnHSF1 in HEK293 cells lowers the PMVK mRNA level.
Previously we have compared the transcriptomes of non-stressed HEK cells with or without expression of a dominant negative HSF1 mutant (dnHSF1) using a two-color 44K Agilent Human Expression Profile Array and found a two- or more fold decrease in the transcript level of 10 genes, amongst which the traditional HSF1 target genes HSP90AA1, HSPA6, DNAJB1 and HSPB1 [9]. One of the other genes of which expression was effected was the phosphomevalonate kinase (PMVK) gene of which the transcript levels decreased fivefold in presence of dnHSF1. To validate the gene expression results obtained from the microarray analysis, we used Q-PCR. Expression of dnHSF1 resulted in a ~ 3 fold decrease of HSP90 mRNA levels, HSPA1A mRNA levels dropped ~ 1.5 times, DNAJB1 mRNA levels declined ~ 9 times and a ~ 3 fold drop of mRNA levels was seen for HSPB1 (Fig. 1), while the levels of PMVK mRNA decreased ~ 10 times (Fig. 1). The decline in PMVK mRNA level correlated with the protein levels of PMVK as the amount of PMVK protein in HEK-dnHSF1 cells decreased when dnHSF1 was expressed (Fig. S1A).

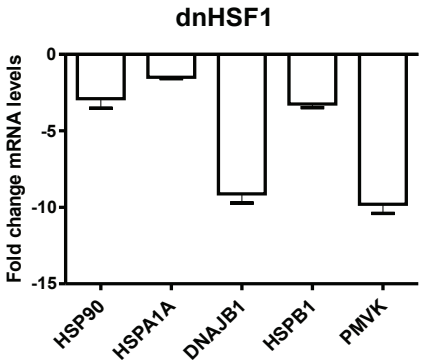

Figure 1 Validation of the dnHSF1 microarray data.
Relative changes in the transcript levels of various genes in HEK-dnHSF1 cells. Changes in HSP90, HSPA1A, DNAJB1, HSPB1 and PMVK mRNA levels relative to GAPDH mRNA levels upon overexpression of dnHSF1 were measured by QPCR. The fold induction of mRNA levels is plotted relative to the mRNA levels in control cells.

Identification of the HSF1 binding site in the human PMVK promoter.
To test which site determined the effect of dnHSF1 on the PMVK promoter we used luciferase reporter constructs driven by the PMVK promoter. Three different lengths of the PMVK promoter were used: -1188, +142; -816, +142 and -620, +142 (depicted in Fig. 2A). DnHSF1 overexpression caused a decrease in the luciferase yield from all three constructs (Fig. 2B), while the activity of the SV40 promoter was not affected by dnHSF1 expression. We used a dominant positive HSF1 (dpHSF1; HSF1Δ202-315) (see Fig. S2 for a schematic representation of the HSF1 expression constructs used in this study) construct to mimic an activated heat shock response and monitored the effect on the activity of the PMVK promoter construct (-1188, +142). The relative luciferase activity increased, indicating that dpHSF1 activates the PMVK promoter (Fig. 2C). The sequence of the PMVK promoter (-1188, -1) did not contain an HSE, but a putative HSE was present in the 5'UTR (+1,

+142). Therefore we placed the 5'UTR sequence upstream of the SV40 promoter (PMVK-5'UTR-SV40 (+1, +142)) and tested the effect of dnHSF1 or dpHSF1 overexpression on the activity of this construct. Figure 2C shows that dnHSF1 inhibited and dpHSF1 increased the activity of the PMVK-5'UTR-SV40 construct, suggesting that the site where HSF1 binds is located

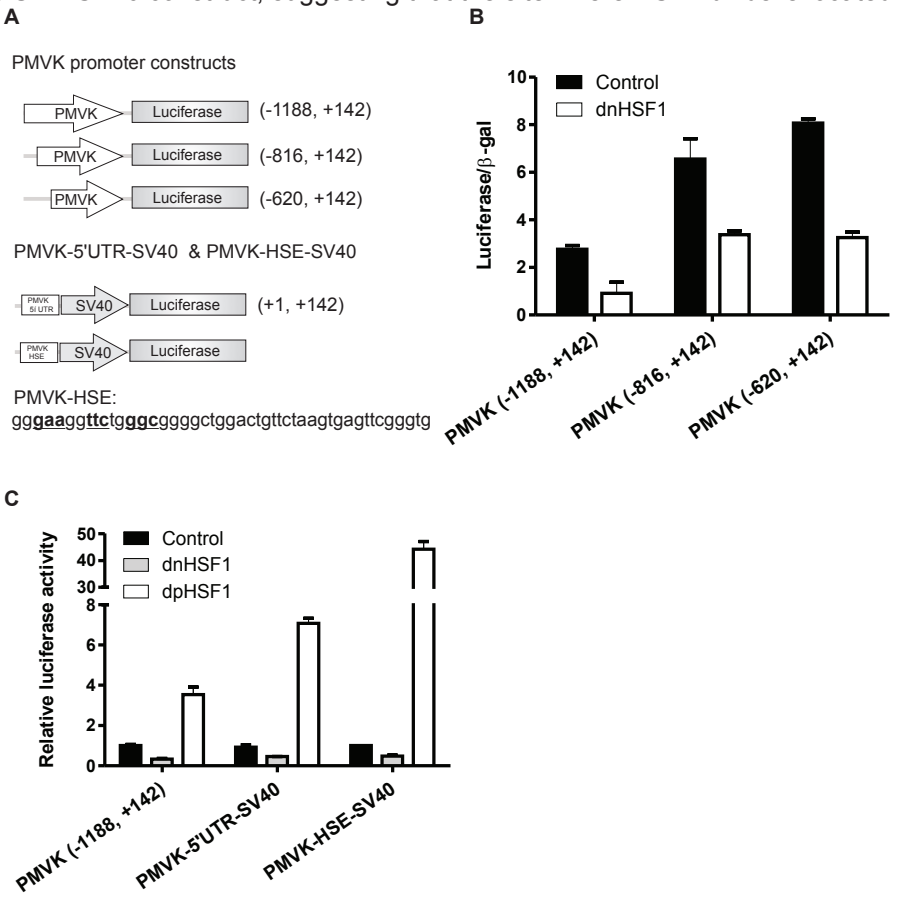

Figure 2 PMVK promoter activity.
A) Schematic representation of the constructs used in the experiments and the sequence of predicted HSE located in the 5'UTR region of PMVK. The nucleotides corresponding to the consensus HSE are shown in bold and underlined.
B-C) The activity of the PMVK reporter constructs is dpHSF1 or dnHSF1 sensitive. HEK-cDNA5 cells were transfected with a mixture of the indicated luciferase reporter, pcDNA5-dpHSF1 or an empty vector and a β-actin-β-galactosidase reporter (7:2:1 ratio). Doxycycline was added 24 hours after transfection and cells were harvested and assayed for reporter gene activities at 48 hours after transfection. HEK-dnHSF1 cells were seeded with or without doxycycline. After 24 hours HEK-dnHSF1 cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 hours after transfection, cells were harvested and assayed for reporter gene activities. Luciferase activities shown are relative to the luciferase activity in control cells, which was set at one.

in this particular region. Further dissection of the PMVK 5'UTR sequence led us to examine $GAAN_2TTCN_2GGC$ as a possible HSE. To test whether this sequences is a target of dnHSF1 or dpHSF1, we constructed PMVK-HSE-SV40 in which the sequence of the PMVK-HSE (Fig. 2A) is placed upstream of the SV40 promoter. DnHSF1 expression decreased the relative luciferase yield from the PMVK-HSE construct, whereas expression of dpHSF1 strongly increased the relative luciferase yield from PMVK-HSE-SV40 (Fig. 2C), suggesting that this particular region of 46 base pairs contains an HSF1 binding site. Note that the PMVK HSE is relatively weak as the response of the Drosophila melanogaster (dm)Hsp70 promoter to dpHSF1 was far higher (Fig. S1B).

Expression of HSF1 mutants affects endogenous PMVK mRNA and protein levels.

The data presented above show that expression of the PMVK gene is inhibited in non-stressed cells by dnHSF1, presumably by binding of dnHSF1 to the HSE located in the region encoding the 5'UTR, i.e. downstream of the promoter. This raises the question whether the expression of the PMVK gene in non-stressed cells is regulated by HSF1. To examine this we used other HSF1 mutants. We tested HSF448 which lacks the C-terminal transactivation domain AD2 but retains the weak transactivation domain AD1 (dnHSF1 lacks both domains, see Fig. S2). HSF448 acts in a weak dominant positive manner under non stress conditions and in a dominant negative way during stress [9]. Another mutant of HSF1 we tested has lysine 80 replaced by glutamine (HSF1-K80Q); this mutation blocks DNA binding ([17]; data not shown). This mutant acts as a dominant negative as it forms heterotrimers with the endogenous HSF1 and thereby also prevents endogenous HSF1 from binding. As control we also overexpressed wild type HSF1. Overexpression of wild type HSF1 also yields some transcriptionally active HSF1 [18]. The effect of overexpression of the HSF1 mutants on the luciferase activity obtained from the HSE driven constructs was as expected. Overexpression of HSF448 or wild type increased the activity of the SV40 promoter driven either by the HSE isolated from the of the Drosophila melanogaster (dm) Hsp70 promoter or the PMVK-HSE, while expression of the HSF1-K80Q mutant had no or little effect (Fig. 3A). Note that the effects of HSF448 or HSF1 WT overexpression on the activity of the dmHsp70-HSE driven construct were stronger than for the PMVK-HSE containing construct, again suggesting that the PMVK is the weaker HSE.

The constructs used in Figure 3A have the isolated HSE placed upstream of the SV40 promoter. In the endogenous situation the PMVK-HSE is located downstream of the transcription start site and functions in the context of other elements and transcription factors regulating promoter activity. We thus also tested the PMVK promoter (-1188 - +142) activity in presence of the different HSF1 mutants. We used the human HSPA1A promoter, a known HSF1 target, as a positive control. As expected, HSPA1A promoter activity

Figure 3 The effect of HSF1 mutants on PMVK levels.

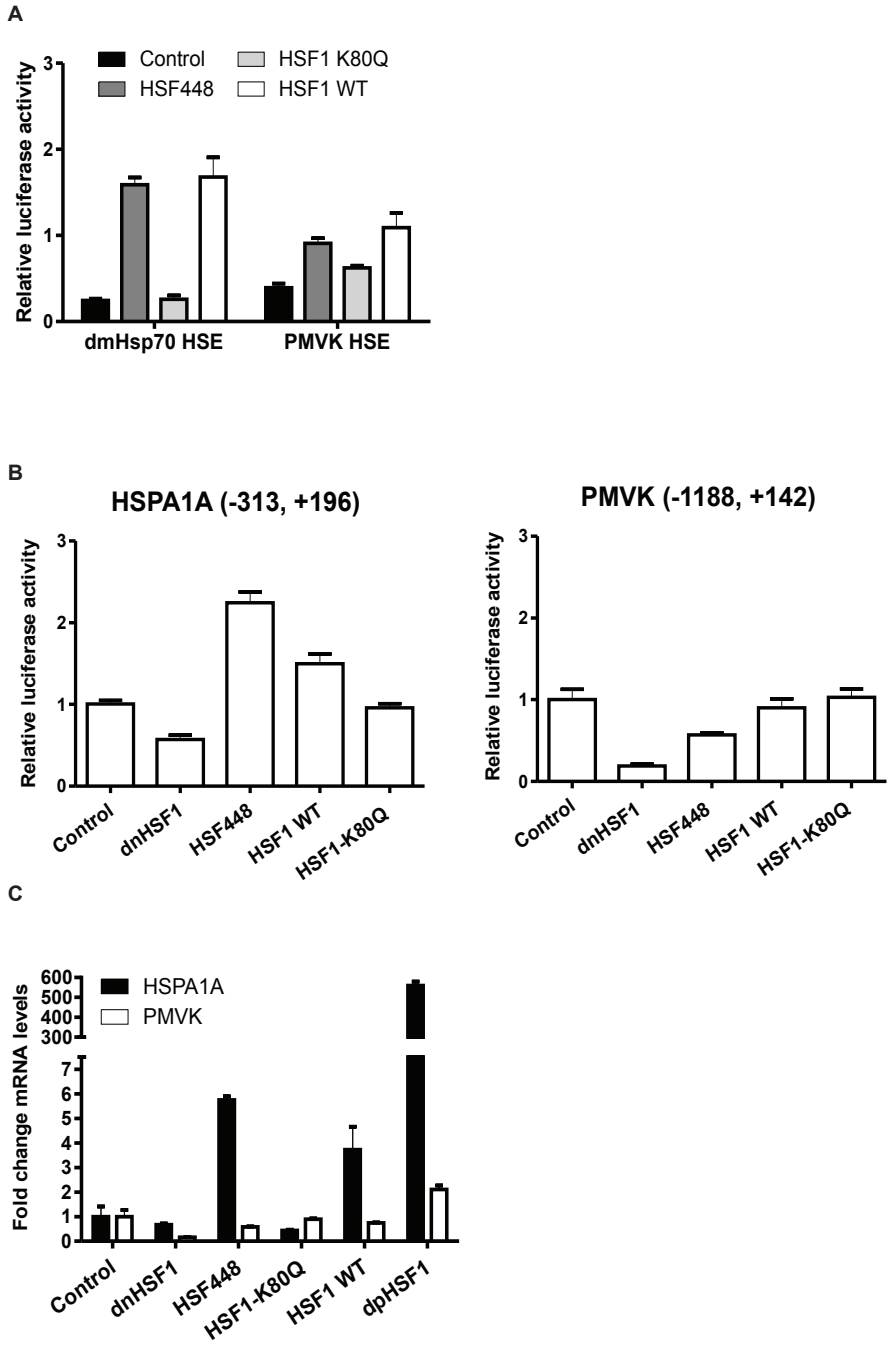

Figure 3 The effect of HSF1 mutants on PMVK levels.
A) The PMVK-HSE acts like a weak HSE. The effects of HSF448, HSF1 WT or HSF1 K80Q overexpresison on the activity of a Drosophila melanogaster Hsp70 HSE containing reporter construct or a reporter construct containing the predicted PMVK HSE. HEK-cDNA5, HEK-HSF448, HEK-HSF1 WT or HEK-HSF1-K80Q cells were seeded in the presence of doxycycline. 24 hours later the cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 hours after transfection, cells were harvested and assayed for reporter gene activities.
B) The PMVK promoter does not act like a typical HSF1 sensitive promoter. HEK-cDNA5, HEK-HSF448, HEK-HSF1 WT or HEK-HSF1-K80Q cells were seeded in the presence or absence of doxycycline. 24 hours later the cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 hours after transfection, cells were harvested and assayed for reporter gene activities. Luciferase activities shown are relative to the luciferase activity in control cells, which was set at one.
C) PMVK and HSPA1A transcript changes in presence of various HSF1 mutants. HEK-cDNA5, HEK-HSF448, HEK-HSF1 WT or HEK-HSF1-K80Q cells were seeded in the presence or absence of doxycycline. 72 hours after seeding, cells were harvested and total RNA was isolated. Transcript levels were measured relative to GAPDH mRNA levels by QPCR. The fold induction of mRNA levels is plotted relative to the mRNA levels in control cells.
the indicated time. Cells were exposed to a heat shock (30', 45°C), harvested at the indicated time point after heat shock, and subjected RT-PCR analysis to investigate the effect of HEK-dnHSF1 on XBP1 splicing after heat stress. C) XBP1s levels in heat stressed cells. HEK-cDNA5 cells were exposed to a heat shock of 30' at 45°C or left at 37°C. When heat shocked, cells were allowed to recover for the indicated time before harvesting. To induce ER stress, cells were treated with 5 µg/ml tunicamycin for 90 minutes. After 5 hours recovery cells were harvested. Cell lysates were subjected to SDS-PAGE and levels of XBP1s were determined by western blotting with γ-tubulin as a loading control.

increased in presence of HSF448 and HSF1 WT, while dnHSF1 overexpression caused a decrease in activity and HSF1 K80Q had little effect. The promoter activity of PMVK was inhibited by dnHSF1 but, surprisingly, also by HSF448 overexpression. HSF1-K80Q did not have an effect on the promoter activity of PMVK and HSF1 WT very slightly inhibited (Fig. 3B). The results from the reporter assays reflect the endogenous situation as the endogenous PMVK mRNA levels also decreased in the presence of the HSF448 mutant. HSF1 wild type or HSF1-K80Q had no effect on the PMVK mRNA level, while expression of the dominant positive HSF1 mutant resulted in an increase in PMVK mRNA levels (Fig. 3C). As control, we also looked at the changes in HSPA1A mRNA levels. As expected, these levels increased upon expression of HSF448, HSF1 wild type and dpHSF1. Note that the HSPA1A gene is only poorly active in non-stressed cells (see also below) and that this background activity is largely independent of HSF1 as indicated by the lack of effect of dnHSF1 or HSF1 K80Q. Similarly, the lack of effect of HSF1-K80Q on PMVK expression shows that in non-stressed cells HSF1 is not required for PMVK promoter activity, while the inhibitory effect of both dnHSF1 and HSF448 imply that if HSF1 is bound to the PMVK HSE, then both HSF1 activation domains are required to maintain PMVK promoter activity. Finally, the stimulatory effect of the dpHSF1 mutant shows that HSF1, when active, can activate the PMVK promoter (Fig. 3C).

Binding of HSF1 to the endogenous PMVK promoter

We used a ChIP assay to determine whether HSF1 binds in vivo to the PMVK HSE identified above either in cells expressing dnHSF1 or in wild type cells. As a positive control we assayed HSF1 binding to the human DNAJB1 promoter. In wild type cells low but significant binding of HSF1 to both the DNAB1 and the PMVK HSEs was detected (compare the signals obtained with either no antibody or an antibody against a transcription factor that does not bind). When the amount of DNA binding competent HSF1 was increased by expression of dnHSF1, an increase in occupancy by HSF1 was detected for both the DNAJB1 and PMVK HSEs. These results show that both endogenous HSF1 and overexpressed dnHSF1 bind the PMVK 5'UTR region in non-stressed cells (Fig. 4).

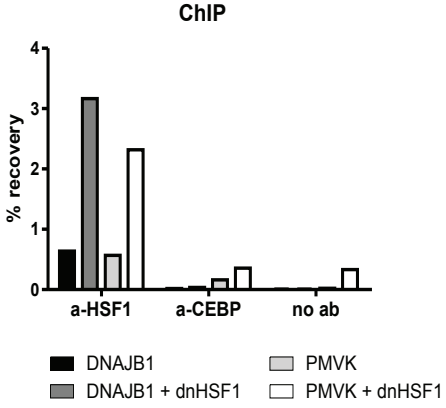

Figure 4 HSF1 binds the PMVK promoter region.
Chromatin immunoprecipitation was performed using an anti-HSF1, or an anti-CEBP antibody as a negative control. Bound chromatin was analyzed by QPCR using a primer set surrounding the HSE of the DNAJB1 promoter and one surrounding the predicted HSE of the PMVK 5' UTR. As a control the ChIP was performed without an antibody.

PMVK expression is not increased in heat shocked cells

If active HSF1 can activate the PMVK promoter, then one would predict that the expression of PMVK is activated when HSF1 is activated, i.e. in cells experiencing cytoplasmic proteotoxic stress. We thus measured PMVK mRNA and protein levels in cells that had recovered for either 6 or 24 hrs from a heat shock. No significant change in PMVK mRNA levels was detected, while the PMVK protein levels decreased. In contrast, HSPA1A mRNA and protein levels did increase sharply upon heat shock (Fig. 5A, B). These results indicate that PMVK is not an HSF1 target during heat shock.

HSF1 is not required for the transcriptional activation of PMVK promoter by sterol depletion.

It has been demonstrated previously that PMVK mRNA levels increase upon depletion of sterol by treatment with the HMG-CoA reductase inhibitor lovastatin [19]. Moreover, HSF1 was shown to be involved in statin induced transcriptional upregulation of endothelial thrombomodulin (TM). Statins were shown to cause NO-dependent dissociation of HSF1 from its chaperone complex followed by nuclear translocation of HSF1 with subsequent binding

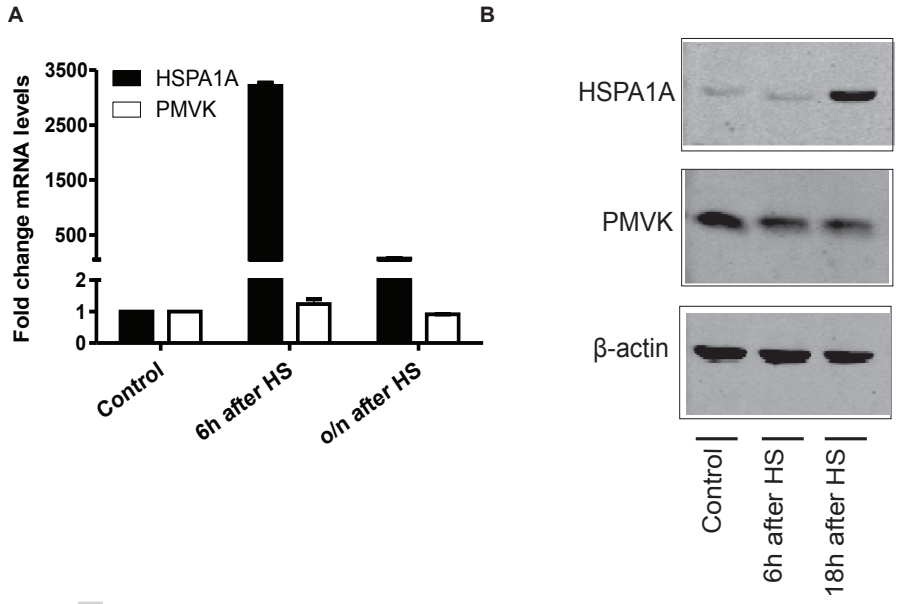

Figure 5 Heat shock response of the PMVK gene.
A) Relative changes in PMVK and HSPA1A transcript levels in heat shocked HEK-cDNA5 cells. HEK-cDNA5 cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. Fold induction of mRNA levels is plotted relative to the transcript level in control cells.
B) HSPA1A, PMVK and β-actin protein levels in HEK-cDNA5 heat stressed cells. HEK cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control), when heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of HSPA1A and PMVK were determined by western blotting using β-actin as a loading control.

to heat shock elements in the TM promoter [20]. Therefore we tested whether the lovastatin induced upregulation of PMVK promoter activity is HSF1 dependent using the luciferase reporter construct driven by the PMVK promoter (-1188, +142). Administration of lovastatin in the presence of charcoal stripped fetal bovine serum did not activate the PMVK promoter. The PMVK promoter activity did increase by about 50% in cells depleted of serum for 24 hours and this effect was enhanced by 1 μM, but not by 10 μM, lovastatin (Fig. 6A). To determine whether HSF1 was involved in the increased activity of the PMVK promoter in serum depleted and lovastatin treated cells, we also assayed the effect of the various HSF1 mutants. HSF1-K80Q had no effect either in control or treated cells. In presence of dnHSF1or HSF448 there was less PMVK promoter activity in untreated cells as expected from the results presented above (Fig. 3B) but the relative increase of the activity in treated cells remained the same (Fig. 6B). PMVK mRNA levels only increased 1.5 fold upon sterol depletion in the presence of lovastatin

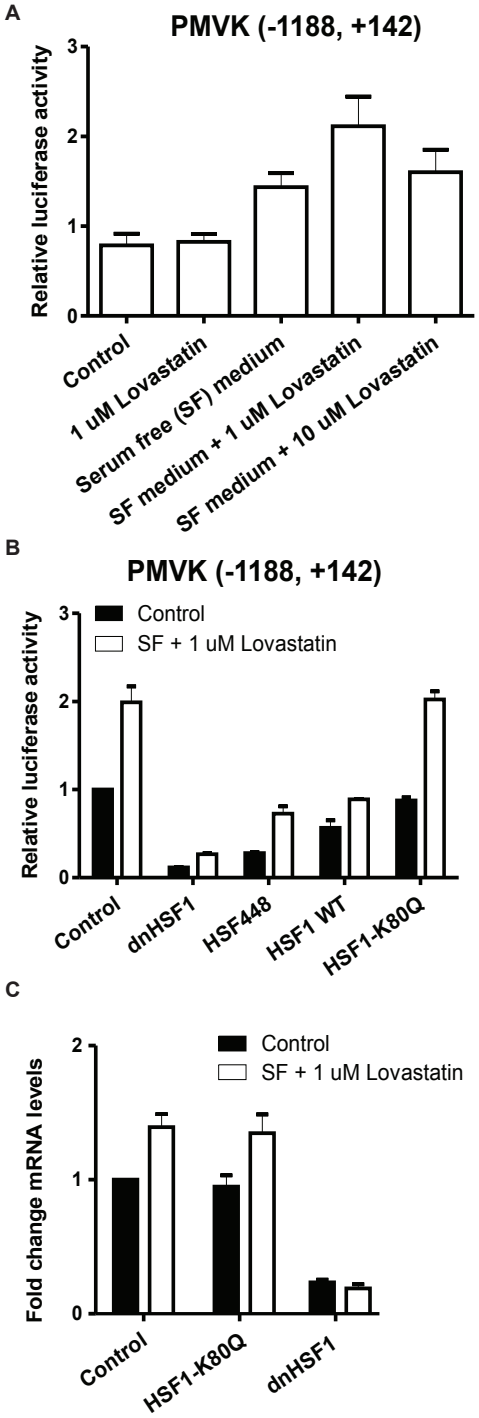

Figure 6 The effect of sterol depletion on PMVK levels.
A) Activation PMVK promoter by sterol depletion. HEK-cDNA5 were seeded in phenol red free medium supplemented 10% fetal bovine serum charcoal stripped, 100 U/ml penicillin, 100 µg/ml streptomycin and GlutaMAX. Cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection cells were exposed to lovastatin in the presence or absence of fetal bovine serum and at different concentrations for 24 hours, then cells were harvested and assayed for reporter gene assay. Luciferase activities shown are relative to that of SV40-Luciferase, which was set at one.
B) Effect of HSF1 mutants on the activation of the PMVK promoter by sterol depletion. HEK-cDNA5, HEK- dnHSF1, HEK-HSF448 or HEK-HSF1-K80Q cells were seeded in the presence or absence of doxycycline. Phenol red free medium supplemented 10% fetal bovine serum charcoal stripped, 100 U/ml penicillin, 100 µg/ml streptomycin and GlutaMAX was used. Cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 24 hours after transfection cells were left untreated or exposed to 1 µM Lovastatin in the absence of fetal bovine serum (SF) for 24 hours, then cells were harvested and assayed for reporter gene assay. Luciferase activities shown are relative to the luciferase activity in control cells, which was set at one.
C) PMVK trancript changes upon sterol depletion. HEK-cDNA5, HEK-dnHSF1 or HEK-HSF1-K80Q cells were treated as described in the legend to Fig. 6B. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. Fold induction of mRNA levels is plotted relative to the transcript level in control cells.

(Fig. 6C). Overexpression of dnHSF1 strongly reduced PMVK mRNA levels, while HSF1-K80Q had no effect on the increase in PMVK mRNA levels upon serum depletion in presence of lovastatin. Together these data show that HSF1 does not play a role in the upregulation of the PMVK promoter upon statin treatment, unlike the TM promoter. It must be noted, however, that sterol depletion by lovastatin treatment in the absence of serum did not result in HSF1 phosphorylation or translocation to the nucleus in our experimental setup (data not shown).

Discussion

Expression of a dominant negative transcription factor is a standard way of showing that the factor plays a role in the transcriptional regulation of a particular gene. In the case of HSF1 we identified a number of genes of which the transcript level was decreased by dnHSF1 expression and which were not otherwise known as HSF1 target genes [9]. Here we have taken a closer look at one of these genes, the PMVK gene. We show that the PMVK gene has an HSE but that this HSE is not located as usual in the proximal upstream promoter region but downstream of the promoter in the region encoding the 5'UTR. Possibly this difference in location explains why the HSF448 mutant that is a weak dominant positive when tested on canonical HSF1 target genes such as HSPA1A inhibits the PMVK promoter. Although the PMVK promoter can be activated by HSF1 as shown by the increase in expression in the presence of exogenous dominant positive HSF1 mutant, we could not find conditions under which endogenous HSF1 regulated transcription of the PMVK gene. The PMVK mRNA levels do not change in heat shocked cells. They do increase somewhat when cells are serum depleted and treated with lovastatin but this increase is HSF1 independent. The PMVK gene is also a target of the androgen receptor [21] and a possible link between the androgen receptor and HSF1 is their common interaction with CHIP [22]. CHIP activates HSF1 and confers protection against apoptosis and cellular stress [23]. Unfortunately HEK293 cells lack the androgen receptor. At present time the PMVK HSE remains in search of a function.

Acknowledgements

We thank Dr. R.P. Dirks for technical support and brainstorming on the matter at the beginning of this project. We thank Dr. H.R. Waterham for the PMVK antibody. This work was financially supported by AgentschapNL (project number IGE03018 and IGE07004, www.agentschapnl.nl).

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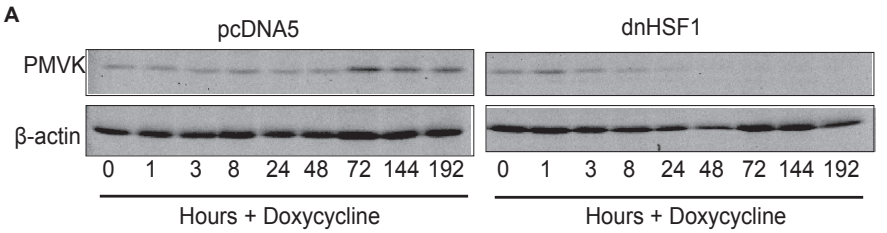
Supplemental tables and figures



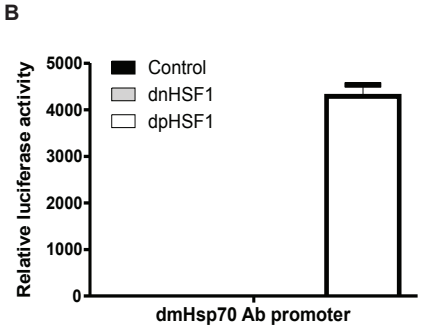


Figure S1 Changes in PMVK protein levels in presence of dnHSF1.
A) HEK-cDNA5 and HEK-dnHSF1 cells were cultered in presence of doxycycline for the indicated periods of time. Cell lysates were subjected to SDS-PAGE and levels of PMVK were determined by western blotting with β-actin as a loading control.
B) The activity of the dmHsp70 reporter construct is dpHSF1 or dnHSF1 sensitive. HEK-cDNA5 cells were transfected with a mixture of the dmHsp70 luciferase reporter, pcDNA5-dpHSF1 or an empty vector and a β-actin-β-galactosidase reporter (7:2:1 ratio). Doxycycline was added 24 hours after transfection and cells were harvested and assayed for reporter gene activities at 48 hours after transfection. HEK-dnHSF1 cells were seeded with or without doxycycline. After 24 hours HEK-dnHSF1 cells were transfected with a mixture of the indicated luciferase reporter and a β-actin-β-galactosidase reporter (9:1 ratio). At 48 hours after transfection, cells were harvested and assayed for reporter gene activities. Luciferase activities shown are relative to the luciferase activity in control cells, which was set at one.

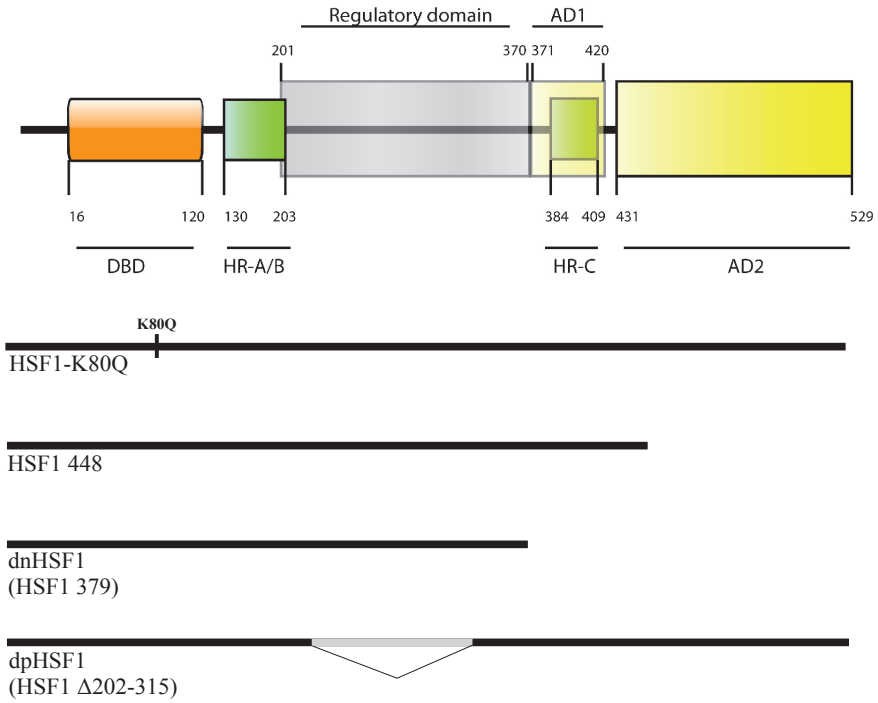


Figure S2 Schematic overview HSF1 mutants.
A schematic representation of the HSF1 expression constructs used in this study.

Chapter 7

HSF1 regulated gene expression in stressed and non-stressed HEK293 cells

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Manuscript in preparation

Abstract

The heat shock response is mainly regulated at the level of transcription by heat shock factor 1 (HSF1). In aging cells HSF1 loses its activity and this failure of HSF1 would interfere with an organism's ability to combat proteotoxic stress and increase the susceptibility to protein folding diseases. To assess the consequences of inactivation of HSF1 in non-stressed and stressed cells, we used Flp-In T-REx-293 cells with tet inducible expression of an HSF1 mutant which is unable to bind DNA (HSF1-K80Q). Exogenous expression of HSF1-K80Q inhibited binding of endogenous HSF1 to the promoter regions of target genes, during non-stress and stress conditions. Microarray and QPCR analysis showed, as expected, that the activation of the known HSF1 sensitive promoters by heat stress was strongly inhibited by HSF1-K80Q expression. These experiments also identified 17 genes of which the transcript levels were decreased in non-stressed cells by HSF1-K80Q, while the transcript levels of 11 genes were higher in the presence of HSF1-K80Q. For 308 genes the transcript levels changed more than 2 fold in heat shocked cells: for 124 genes the transcript levels decreased, while for 184 the levels went up. Of these 184, only 30 were 2-fold or more inhibited by HSF1-K80Q expression and thus HSF1 dependent. HSF1-K80Q inhibited recovery from heat shock and some stress induced transcripts remained high in cells allowed to recover from heat shock for 24 hrs. Surprisingly, HSPA1A and HSPA6 mRNA levels were increased 24 hrs after heat shock in cells expressing HSF1-K80Q, while, as expected, the mRNA levels were not increased after 6 hrs of recovery. Apparently there is a second wave of stress induced transcription, which does not require HSF1.

Introduction

Cells respond to cytoplasmic proteotoxic stress by producing heat shock proteins (HSP). This response is called the heat shock response (HSR) and is known as an important mechanism of protection against cytoplasmic proteotoxic stress. The heat shock response is mainly regulated at the level of transcription by heat shock factor 1 (HSF1). The trimeric and transcriptionally active form of HSF1 possesses an increased affinity for the heat shock element (HSE) [1]. During aging, the activity of HSF1 declines. The protein is still present but can somehow no longer be activated. This aging-related failure of HSF1 interferes with an organism's ability to combat proteotoxic stress which results in increased susceptibility to protein folding diseases [2-7]. Furthermore, with accumulating evidence showing that HSF1 also regulates gene expression under non-stress conditions, a decline in HSF1 activity may already cause phenotypic defects in the absence of exogenous stress [8]. Previously we have used an HSF1 mutant retaining the DNA binding domain but lacking the activation domain (dnHSF1) to inhibit HSF1 activity in non-stressed cells [9]. Expression of dnHSF1 reduced the expression level of only 10 genes more than two-fold and did not increase the expression level of any gene more than two-fold. In contrast, HeLa cells treated with siRNA directed against HSF1 showed changed expression levels of 378 genes in the absence of stress [10] where 80% of the affected genes showed increased transcript levels. A comparison of the transcriptome of MEF HSF1 -/- with that of MEF WT cells resulted in 49 genes (19 related to immune response) that were expressed at reduced levels in MEF HSF1 -/- cells [11]. HSF1 thus seems to act as a repressor of transcription in the case of HeLa cells and the loss of HSF1 releases repression while expression of dnHSF1, which remains bound to the DNA, might maintain it. The difference between depleting HSF1 and expressing a dominant negative mutant may also be partly due to a secondary effect: depletion of HSF1 would free the chaperones which are usually complexed with HSF1 while dnHSF1 might capture more chaperones. Another difference is the involvement of HSF2. Recently it has been postulated that HSF2 also plays a role in the proteotoxic stress response and is recruited to HSF1 responsive promoters as a heteromer with HSF1. HSF1 is the main regulator of the heat shock response, but HSF2 binds to the promoters of HSF1 target genes as well. HSF2 only binds in the presence of HSF1 and an intact DNA binding domain of HSF1 is required [12].

The aging cell differs from the HSF1-/- cells in that the cell still contains HSF1, although not active, and differs from the dnHSF1 cells in that HSF1 is no longer bound to its target promoters. We have started to build a model system that is a closer mimic of the aging cell, i.e. a cell that still expresses HSF1, but in an inactive form. To that end we made an HSF1 mutant in which lysine 80 in the DNA binding region is replaced by glutamine (HSF1-

K80Q), thus impairing DNA binding [13]. The HSF1-K80Q expression construct was used to make a stable Flp-In T-REx-293 cell line with tet inducible expression of HSF1-K80Q.
We show here that overexpression of HSF1-K80Q inhibited binding of HSF1 to its target promoter regions, during non-stress and stress conditions. Microarray analysis identified 29 genes of which the transcript level was affected by HSF1-K80Q expression in non-stressed cells; for 17 the transcript levels decreased, while for 11 the transcript levels increased. As expected, expression of HSF1-K80Q blocked the heat shock induced increase in transcript levels of known HSF1 dependent genes. However, most of the stress induced changes in the transcriptome were HSF1 independent.
Surprisingly, we noticed that the HSPA1A and HSPA6 mRNA levels were increased after 24 hrs recovery from a heat shock in the HSF1-K80Q expressing cells, while, as expected, they were not increased after 6 hrs of recovery. Apparently there is a second wave of stress induced transcription, which does not require HSF1.

Materials and Methods

Cell culture
Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-dnHSF1, HEK-HSF1-K80Q, HEK-HSF1-WT and HEK-cDNA5 that carry a single copy of the tetracycline-inducible plasmids pcDNA5-dnHSF1, pcDNA5- HSF1-K80Q, pcDNA5-HSF1 WT and pcDNA5-FRT/TO, respectively. The cells were cultured at 37°C in the presence of humified 5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments.

Plasmid Construction, transfections and reporter gene assays
The dnHSF1 expression vector and the dmHSP70 and HSP70A1A reporter constructs have been described earlier [9]. The pcDNA5-HSF1 silent mutation (HSF1 WT) and the pcDNA5-HSF1-K80Q mutant were made by performing site-directed mutagenesis on pcDNA5-HSF1 with respectively the HSF1 sil.mut and the HSF1-K80Q primers, listed in Supplemental Table 1. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with 0.2 µg plasmid per well. 20 ng CMV-β-galactosidase was used as a transfection efficiency control. At 24 hours after transfection doxycyclin was added. At 48 hours after transfection cells were

harvested or exposed to heat shock (30' 45°C).
Cells were harvested and lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 20 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus; Tropix). After 30 minutes incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 20 µl cell lysate was mixed with 50 µl luciferin solution (Promega) and luminescence was measured with the Lumat luminometer. All reporter gene assays were performed in triplicate. Relative activities of luciferase reporter genes were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies.

Western blot analysis
Cell pellets were homogenized in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4x sample buffer (200 mM Tris–HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. Protein samples were separated in 10% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1:1,000 dilution, monoclonal HSP90 antibody (610418, BD Biosciences) at a 1:1,000 dilution, polyclonal HSPB1 antibody, obtained from Dr. A. Zantema, at a dilution of 1:400, monoclonal HSPA1A antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye® 800CW conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32210, respectively; LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

QPCR analysis
Cells were harvested after the treatments and at the times indicated, washed twice with ice-cold PBS and after washing the cells, RNA was isolated with TRIzol (Invitrogen) according to manufacturer's recommendation. 1 µg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 µM oligo(dT) were added to a total volume of 20 µl. Reverse transcription was performed

for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with Power SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in Supplemental Table 1.

Chromatin Immunoprecipitation

HEK-XBP1s or HEK-cDNA5 cells were cultured for 24 h in the presence of doxycycline. Chromatin immunoprecipitation was performed as described in [14] except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES-KOH pH 7.6, 140 mM NaCl, 1mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor complete). Sonicated chromatin was centrifuged for 5 min at 4°C and then incubated overnight in incubation buffer (final concentration; 12 mM HEPES-KOH pH 7.6, 90 mM NaCl, 0.6 mM EDTA pH. 8.0, 0.09% SDS, 0.6% Triton X-100, 0.1% BSA) together with purified anti-HSF1 antibody (SPA-901; Stressgen) and protein A/G beads (Santa Cruz Biotechnology). Negative control without adding antibody was included. Beads were washed six times with different buffers at 4°C: twice with 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 150 mM NaCl, HEG (1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES-KOH pH 7.6), once with the same buffer but with 500 mM NaCl, once with 0.25 M LiCl, 0.5% NaDOC, 0.5% NP-40, HEG and twice with HEG. Precipitated chromatin was eluted with 400 µl of elution buffer (1% SDS, 0.1 M NaHCO3), incubated at 65°C for 4 h in the presence of 200 mM NaCl, phenol extracted and precipitated with 20 µg of glycogen at -20°C overnight. ChIP experiments were analyzed by qPCR. Efficiency of ChIP was calculated as percentage of input. The primers used are listed in Supplemental Table 1.

EMSA

HEK-HSF1-K80Q or HEK-HSF1 WT cells were cultured for 24 h in the presence or absence of doxycycline and subsequently heat shocked for 30 minutes at 45°C. Cells were immediately harvested and nuclear extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Extracts were aliquoted and stored at -80°C. Oligonucleotide probes were end-labeled with ^{32}P. The sequences of the HSE oligonucleotides used in EMSA are listed in Supplemental Table 1. The EMSA protocol was adapted from [15, 16]. A mixture containing 5 µg nuclear extract and 3 µg poly dIdC in binding buffer (20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll, 1X PhosSTOP (Roche)) was incubated for 20 minutes on ice.

0.01 pmol radiolabeled oligonucleotide was added and again the samples were incubated for 20 minutes on ice. DNA-protein complexes were separated on a pre-run 4% polyacrylamide gel in 0.25x TBE with recirculation of the buffer. The gel was dried and signals were visualized using a Phosphor Imager.

Microarray analysis

HEK-pcDNA cells and HEK-HSF1-K80Q cells were treated with doxycyclin for 48 hours. The transcriptomes of HEK-pcDNA cells and HEK-HSF1-K80Q cells, HEK-HSF1-K80Q 6h after heat shock versus unstressed HEK-HSF1-K80Q cells or HEK-pcDNA5 6h after heat shock versus unstressed HEK-pcDNA5 cells were compared. Total RNA was isolated using Trizol according to the manufacturer's instructions (Invitrogen) and copied into Cy3-labeled or Cy5-labeled cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS, and reverse labeled for the repeat array. Labeled cRNA samples were hybridized to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). We used a cut-off level of 2-fold changed expression and an arbitrarily chosen signal cut-off of > 50.

Results and Discussion

Characterization HEK- HSF- K80Q cell line

In the HSF1-K80Q mutant lysine 80 in the DNA binding region is replaced by glutamine and because of this mutation HSF1 loses its binding activity [9, 13]. Exogenously expressed HSF1-K80Q is expected to trimerize with endogenous HSF1, resulting in a strong reduction of binding competent HSF1 trimers. We tested whether this indeed happens. Only a weak signal of HSF1 binding to the HSE was detected using nuclear extracts of either unstressed or heat shocked cells expressing HSF1-K80Q. Using nuclear extracts from cells overexpressing wild type HSF1 (HSF1 WT) did result, as expected, in increased binding of HSF1 to the HSE even when cells were unstressed (Fig. 1A). The bandshift shown in figure 1A could be supershifted by an antibody to HSF1, indicating that it is indeed HSF1 that was bound (data not shown). Extracts from heat shocked cells showed a more intense bandshift signal and thus an increase in binding competent HSF1. Expression of HSF1-K80Q blocked this increase. The loss of binding of HSF1 to the HSE in the presence of HSF1-K80Q was confirmed by chromatin immunoprecipitation (ChIP) (Fig. 1B). In control cells, i.e. HEK-HSF1-K80Q cells cultured in the absence of doxycycline, HSF1 was bound to the HSPA1A or HSPA6 promoter region 2 hours after heat shock. The binding of HSF1 is transient and as expected we did not observe HSF1 binding 18 hours after heat stress. When HSF1-K80Q expression was induced, no bound HSF1 could be detected either 2 hours or 18 hrs after heat shock.

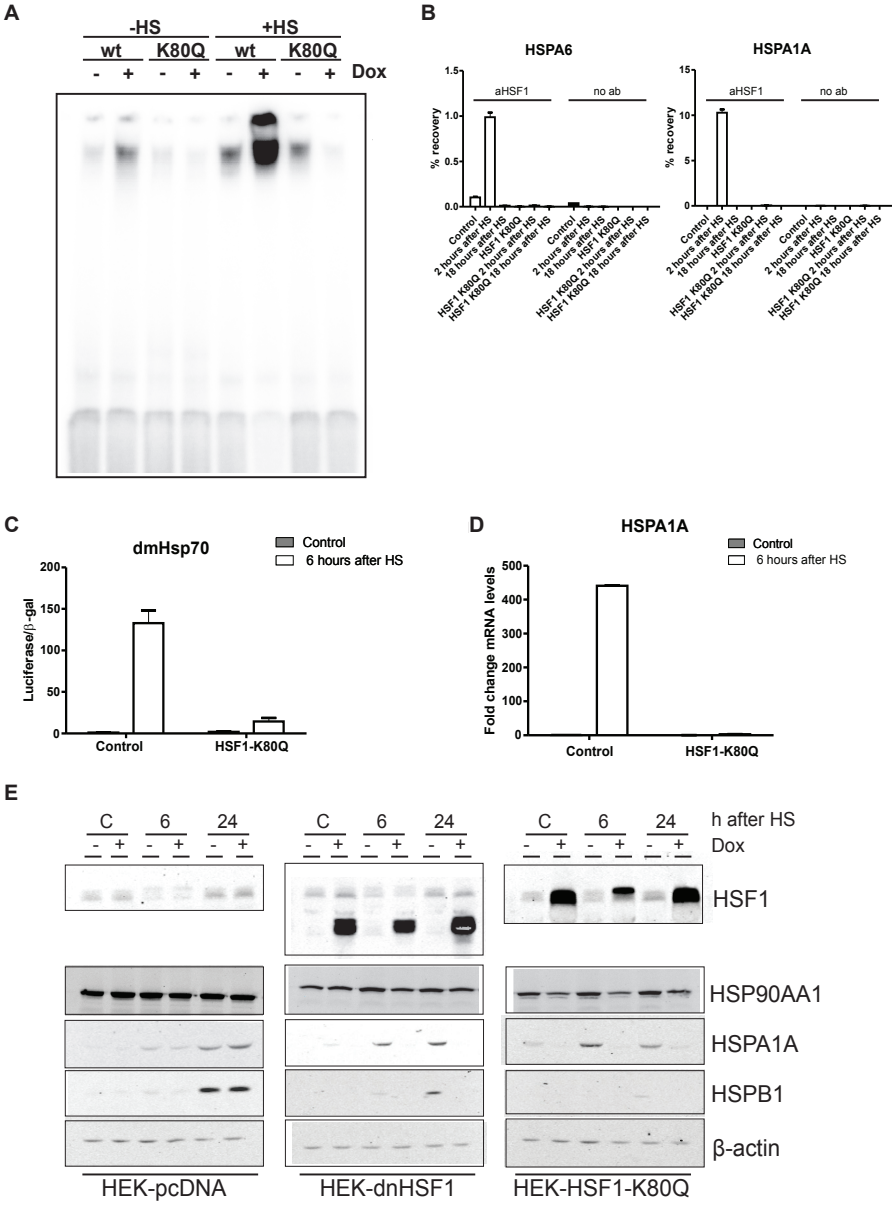


Figure 1 Characterization of HEK-HSF1-K80Q cells.
A) Nuclear extracts were made of HEK-HSF1 WT cells and HEK-HSF1 K80Q cells either non-stressed (-HS) or exposed to heat shock (30' 45°C, +HS). An electrophoretic mobility shift assay (EMSA) was performed with a ds oligo for the HSE sequence. Where indicated (+ Dox), doxycyclin was added to induce expression of either HSF1 WT or HSF1-K80Q.
B) Chromatin immunoprecipitation (ChIP) using nuclear extracts from control and HSF1 K80Q expressing cells was performed with an HSF1 antibody or no antibody added. Bound chroma-

tin was analyzed by QPCR using a primer set surrounding the HSE of the HSPA6 or HSPA1A promoter. Cells were either non-stressed or harvested 2 hrs or 18 hrs after heat shock, as indicated.
C) The effect of HSF1-K80Q overexpresison on basal and heat shock-induced activity of the Drosophila melanogaster Hsp70 promoter. HEK293 cells carrying a stably integrated copy HSF1-K80Q or an empty vector were cultured in the presence of doxycycline. Cells were transfected with a mixture of the Drosophila melanogaster Hsp70-luciferase reporter and pCMV-β-galactosidase. At 48 hours after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for 6 hours and harvested. Hsp70 promoter activities were determined by dividing firefly luciferase values by β-galactosidase values to correct for varying transfection efficiencies. The results are the average of four independent transfections (standard deviations are indicated by error bars).
D) HSF1-K80Q blocks the heat shock induced increase in HSPA1A transcript. HEK-HSF1-K80Q cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells.
E) HSF1-K80Q blocks the heat shock induced increase in HSPA1A and HSPB1. HEK-pcDNA, HEKdnHSF1 or HEK-HSF1-K80Q cells were cultured in the presence or absence of doxycycline and exposed to a heat shock of 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of HSF1, HSP90, HSPA1A, and HSPB1 were determined by western blotting. β-actin was used as a loading control.

To confirm that expression of HSF1-K80Q inhibits HSF1 driven promoters, we used a luciferase reporter constructs driven by the D. melanogaster Hsp70 promoter. The heat shock-mediated induction of D. melanogaster Hsp70 promoter activity was almost completely abolished by expression of HSF1-K80Q (Fig.1C). Similarly, the heat shock-mediated increase of human HSPA1A mRNA six hours after heat shock was abolished by HSF1-K80Q (Fig. 1D). The increase in endogenous HSPA1A, HSP90AA1 and HSPB1 protein levels after heat shock was also completely inhibited in the presence of HSF1-K80Q (Fig 1E), just as it was by dnHSF1. Together these data show that HSF1-K80Q acts as a dominant negative mutant and blocks the HSF1 directed transcriptional heat shock response.

Transcriptome changes in the presence of HSF1-K80Q

If HSF1 plays a role in the absence of stress, then expression of a non DNA binding mutant could change the transcriptome. We used microarrays to analyze the effect of expression of HSF1-K80Q on the transcriptome in the absence of stress. As overexpression of the HSF1 protein may have secondary effects, for example by sequestering chaperones, we also looked at the effect of overexpressing wild type HSF1on the transcriptome of non-stressed cells. Table 1 shows the list of the 28 genes of which the transcripts level changed at least two fold upon expression of HSF1-K80Q in non-stressed cells but of which the level was not significantly affected by expression of wild type HSF1 (see also Supplemental Table 2). For 17 genes we noted a decrease in transcript level, of these, 2, LY6K and VCX2, had an increased transcript level in heat shocked cells. None of the 11 genes of which the tran-

script levels increased in non-stressed HSF1-K80Q cells, were heat shock responsive. The increase in the level of the transcript of the PCK2 gene, which encodes mitochondrial phosphoenolpyruvate carboxykinase 2, was confirmed by QPCR (Fig. 2A).

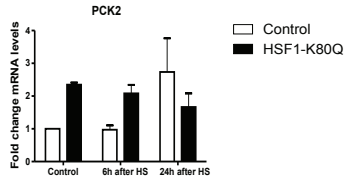
Table 1 Genes of which the transcript level changes at least 2-fold in non-stressed HSF1-K80Q cells.

Down		Up	
Gene name	**Acc. Nr.**	**Gene name**	**Acc. Nr.**
BFSP1	NM_001195	ASNS	NM_001673
C20orf201	NM_001007125	CTH	NM_001902
CGA	NM_000735	JUB	NM_032876
CRLF1	NM_004750	NUPR1	NM_0010424
FERMT1	NM_017671	PCK2	NM_004563
KRT222	NM_152349	RNF125	NM_017831
LOC100292909	XM_002345507	SEPP1	NM_005410
LY6K	NM_017527	SLC7A11	NM_014331
NPTX2	NM_002523	STC2	NM_003714
PLEKHG4	NM_015432	TPK1	NM_022445
PPAPDC1A	NM_001030059	WDR13	NM_017883
RAI2	NM_021785		
SLC16A5	NM_004695		
SYTL5	NM_001163335		
UBE2C	NM_181803		
VCX2	NM_016378		
ZNF334	NM_199441		

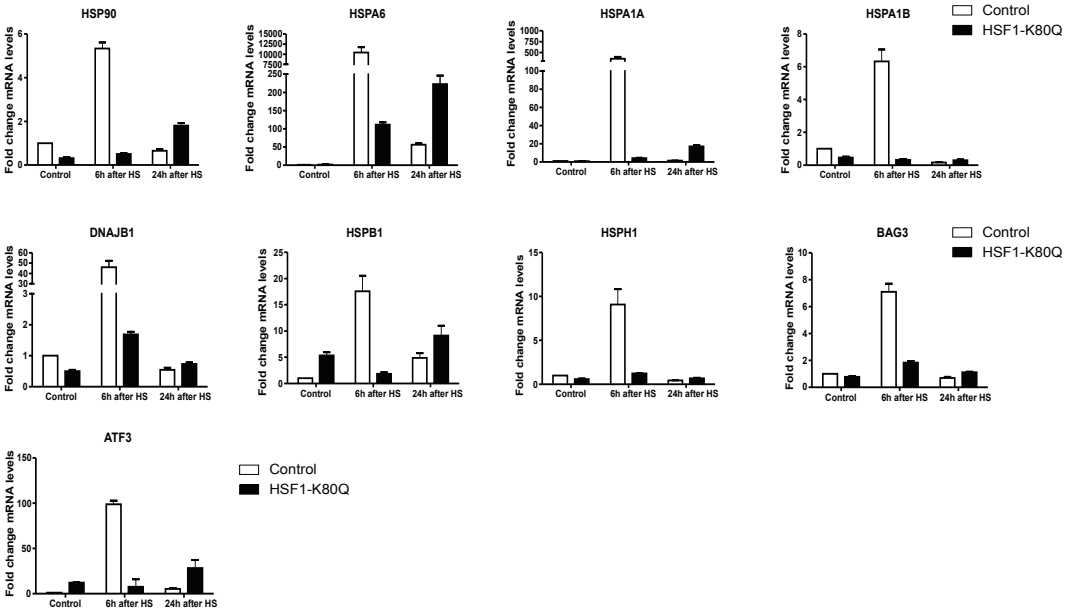
The classical role of HSF1 is transcription activation in heat stressed cells. In HEK293 cells allowed to recover from a heat shock for 6 hrs, we found in an increase of at least two fold in the transcript levels of 184 genes (see below). Expression of HSF1-K80Q inhibited the increase in transcript level of 30 of these genes by at least two fold (Table 2, Supplemental Table 3). Amongst these genes are the canonical HSF1 target genes, such as HSPA1A/B, HSPA6, DNAJB1, HSPH1, HSP90AA1, BAG3 but also ATF3 (we also included HSPB1 and SERPINH1 in this class even though they failed to meet the arbitrary 2-fold cut-off). We confirmed the inhibitory effect of the expression of HSF1-K80Q on the increase in transcript levels of these genes (Fig. 2B). Curiously, according to the QPCR data, but not the microarray data, the transcript levels of the ATF3 and HSPB1 genes were higher in non-stressed cells expressing HSF1-K80Q.

Figure 2 Relative changes in transcript levels of various genes in stressed and non-stressed HEK-HSF1-K80Q cells.
(A-C) HEK-HSF1-K80Q cells were cultured in the presence (HSF1-K80Q) or absence of doxycycline (Control) and exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells.

A


PCK2
Fold change mRNA levels
Control
6h after HS
24h after HS
Control
HSF1-K80Q

B


HSP90
HSPA6
HSPA1A
HSPA1B
DNAJB1
HSPB1
HSPH1
BAG3
ATF3
Fold change mRNA levels
Control
6h after HS
24h after HS
Control
HSF1-K80Q

C

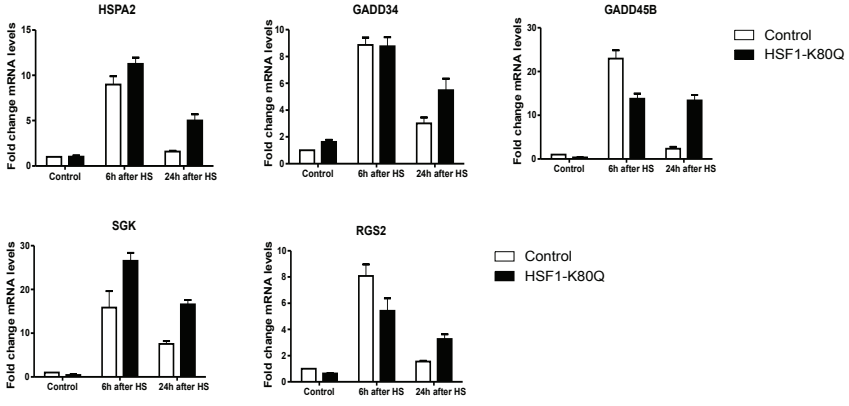

HSPA2
GADD34
GADD45B
SGK
RGS2
Fold change mRNA levels
Control
6h after HS
24h after HS
Control
HSF1-K80Q

Table 2 Genes of which the increase in transcript level in heat stressed cells was inhibited at least 2-fold by HSF1-K80Q.

Down		Up	
Gene name	**Acc. Nr.**	**Gene name**	**Acc. Nr.**
ATF3	NM_001674	HSPA6	NM_002155
BAG3	NM_004281	HSPB1*	NM_001540
BU532663 (SNORD3D)	BU532663	HSPH1	NM_006644
C17orf67	ENST00000397861	JUN	NM_002228
C22orf43	NM_016449	LOC100130288	BC043212
CACYBP	NM_014412	LOC100133337	XM_002343495
CCDC121	NM_024584	LOC387763	NM_001145033
CRYAB	NM_001885	MRPL18	NM_014161
DEDD2	NM_133328	NXT2	NM_018698
DHDH	NM_014475	SERPINH1*	NM_001235
DNAJB1	NM_006145	UBB	NM_018955
DNAJB4	NM_007034	UBC	NM_021009
DUSP1	NM_004417	USP51	NM_201286
HSP90AA1	NM_005348	USPL1	NM_005800
HSPA1A	NM_005345	ZFAND2A	NM_182491

*did not quite meet the 2-fold cut-off

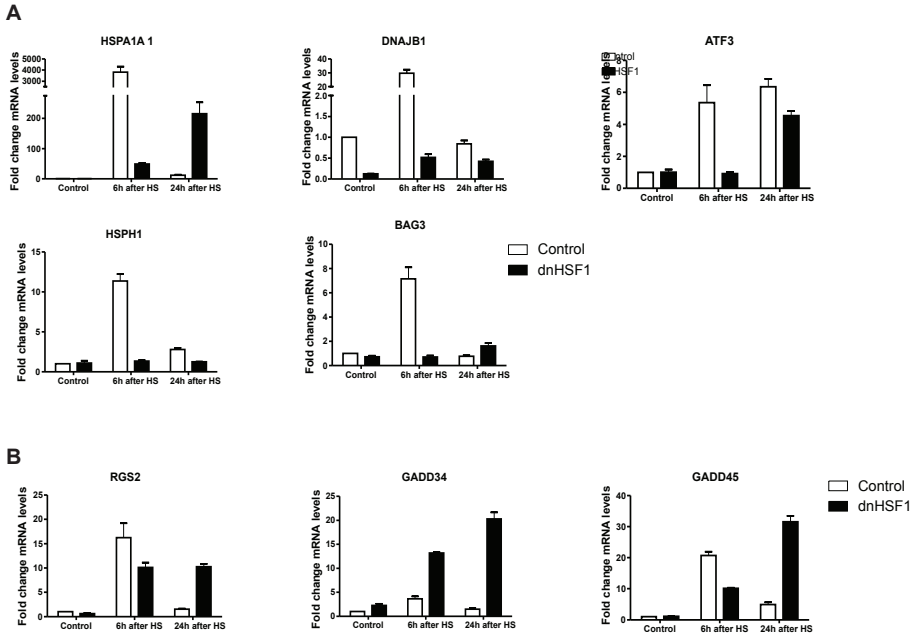


Figure 3 Relative changes in transcript levels of various genes in stressed and non-stressed HEK-dnHSF1 cells.
(A-B) HEK-dnHSF1 cultured in the presence (dnHSF1) or absence of doxycycline (Control) cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (Control). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells.

The expression of HSF1 dependent chaperones serves as a feedback mechanism to dampen the heat shock response [17]. As in the HSF1-K80Q expressing cells the expression of these chaperones is inhibited, we tested whether expression of HSF1-K80Q also affects recovery from a heat shock as assessed by the level of transcripts of heat shock activated genes in cells that were allowed to recover for 24 hrs from the heat shock. Of some HSF1 target genes (for example HSPA1A, HSPA6, ATF3) the transcript levels were higher in HSF1-K80Q cells than in control cells, while for other HSF1 target genes (for example HSPA1B, DNAJB1) the transcript levels were not significantly different between wild type and HSF1-K80Q expressing cells (Fig. 2B).

Most of the transcriptome changes in heat shocked cells were HSF1 independent, that is the change was not inhibited but sometimes even enhanced by the expression of HSF1-K80Q (Supplemental Table 3). We confirmed the changes in RGS2 (regulator of G-protein signaling 2), GADD34 (PPP1R15A), GADD45B, HSPA2 and SGK1 (serum/glucocorticoid regulated kinase 1) transcript levels. The transcript levels of the HSPA2 and SGK1 genes were higher in HSF1-K80Q expressing cells 6 hrs after heat shock, that of the GADD34 gene was equal and those of the RGS2 and GADD45B genes were lower, but the extent of inhibition by HSF1-K80Q was far less than for the canonical HSF1 target genes. In all cases the transcript levels were much higher in HSF1-K80Q expressing cell than in control cells 24 hrs after heat shock (Fig. 2C).

The persistence of stress induced transcripts in HSF1-K80Q cells is a sign that HSF1-K80Q, as expected, recuperate more slowly from stress. The increase in transcript levels of canonical heat shock genes in HSF1-K80Q cells 24 hrs after heat shock (Fig. 2B) is rather unexpected, as HSF1-K80Q should block HSF1 dependent transcriptional activation. We therefore tested whether the increase in the level of some of the canonical HSF1 targets 24 hrs after heat shock is also seen in cells expressing dnHSF1. As shown in Fig. 3, this is indeed the case: in the presence of dnHSF1 the HSPA1A and BAG3 transcript levels were significantly higher 24 hrs after heat shock. The levels of the DNAJB1 and the HSPH1 transcripts did not show this 24 hr response in dnHSF1 expressing cells, just as they did not in HSF1-K80Q expressing cells. Similarly, the levels of the non-HSF1 target genes, GADD34 and GADD45B, did not decrease in dnHSF1 expressing cells. Hence, the response of at least these genes is the same in dnHSF1 and HSF1-K80Q expressing cells.

These data indicate that the activity of some transcriptions factors, which normally decays after heat shock, is maintained when HSF1 activity is blocked. One possibility is that the level of endogenous HSF1 is increased in cells recovering from heat shock, which would decrease the efficacy of the dnHSF1 and HSF1-K80Q mutants. However, no change in the endogenous level of HSF1 in dnHSF1 expressing cells could be detected (Fig. 1E; note that the endogenous HSF1 and the HSF1-K80Q cannot be distinguished on

SDS PAGE). The GADD34 gene is a target of ATF4 [18] and we thus tested whether the level of ATF4 was affected by expression of either dnHSF1 or HSF1-K80Q. As shown in Fig. 4, it was not. At this moment we do not know which transcription factors are responsible for this second wave of stress induced transcription. The activity of the HSPA1A promoter has been reported to rely also on NF-Y which interacts with CCAATTG box [19-21]. We are presently testing whether NF-Y is also required for the increased transcript levels of HSF1 target genes in HSF1-K80Q or dnHSF1 expressing cells.

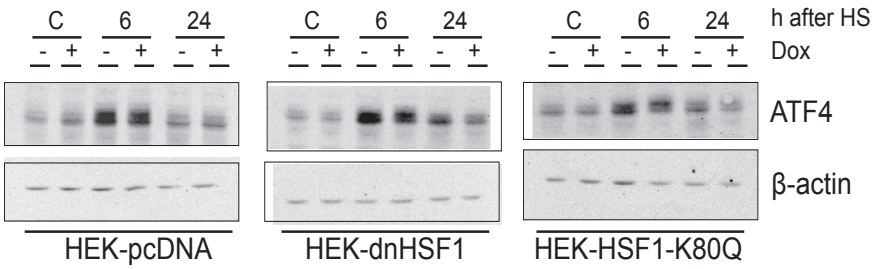


Figure 4 ATF4 protein levels do not change in presence of HSF1 mutants.
HEK-cDNA5, HEK-dnHSF1 and HEK-HSF1-K80Q cells were cultured in the presence or absence of doxycycline and exposed to a heat shock of 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of ATF4 were determined by western blotting. β-actin was used as a loading control.

Table 3A Transcriptome changes in HSF1-K80Q HEK293 cells.

	Up	Down
Non-stressed, 28 changed		
HEK-HSF1-K80Q	11	17
6h heat shock, 308 changed		
HEK-pcDNA	184 (30 inhibited by HSF1-K80Q)	**124**

Comparison of transcriptome changes in the presence of HSF1-K80Q or siRNA HSF1

As outlined in the introduction, a lack of HSF1, due to expression of siRNA against HSF1, may have a different effect than expression of a non-DNA binding HSF1 mutant. We therefore compared our microarray data with those in the literature using siRNA against HSF1 in HeLa cells [10] (Table 3B). For the 17 out of the 28 genes of which the transcripts levels were changed in non-stressed HSF1-K80Q expressing cells, we could find data in the HSF1 siRNA treated HeLa cells (Supplemental Table 4A). For 16

of these the transcript levels were not significantly affected by the siRNA treatment; one (CRLF1, cytokine receptor-like factor 1) was downregulated as it was in HEK HSF1 K80Q cells. When we compared the data for heat shocked Hela and HEK293 cells we found only 19 genes that were heat shock responsive in both HeLa and HEK293 cells (note that the exact heat shock conditions do differ). Of these, one gene (ZNF264) had a lower transcript level. This regulation appeared to be independent of HSF1 activity. Of the 18 genes of which the transcript levels increased in both heat shocked HeLa and HEK293 cells, 7 were clearly HSF1 dependent, the activity of 3 was inhibited significantly only by HSF1 siRNA, that of 4 only by HSF1-K80Q and 4 appeared to be not regulated by HSF1 (Table 3B, C and figure 5; Supplemental Table 4B).

There is almost no overlap between the genes of which the transcript level changed significantly in non-stressed cells upon siRNA HSF1 treatment or upon overexpression of HSF1- K80Q. Either the genes controlled by HSF1 in the non-stressed state are largely cell specific or the effect of deleting HSF1 from a cell is significantly different from blocking HSF1 activity by overexpression of a non-DNA binding HSF1 mutant. To distinguish between these possibilities the comparison must be made using the same cells. The HSF1 dependent stress response is much less cell specific: most of these genes are the traditional HSF1 target genes such as DNAJB1. Curiously,

Table 3B Comparison of the transcriptome changes in HSF1-K80Q HEK293 and siHSF1 HeLa cells.

Gene name	Up	Down	No change
non-stressed, out of 28 changed in HEK293, 17 also on HeLa array			
Up in HEK293 array & also on HeLa siRNA array	0	0	8
Down in HEK293 array & also on HeLa siRNA array	0	***1 (CRLF1)***	8
6 h heat shock, out of 308 changed in HEK293, 163 also on HeLa array			
Up in HEK293 array & also on HeLa siRNA array	***18****	3	98
inhibited both K80Q and siRNA	*7*		
inhibited siRNA only	3		
inhibited K80Q only	4		
inhibited neither K80Q nor siRNA	***4***		
Down in HEK293 array & also on HeLa siRNA array	0	***1 (ZNF264)***	43
inhibited neither K80Q nor siRNA		***1 (ZNF264)***	

*The HSF1 target genes HSPA1A, HSPB1 and SERPINH1 were included even though they did not meet the cut-off.
In bold and italic: Overlap HeLa and HEK293 array

and presumably due to the stress conditions used, the best known HSF1 regulated gene, HSPA1A, is not quite two-fold upregulated in heat shocked HeLa cells and its activity is only little affected by HSF1 siRNA treatment [10]. The common heat shock response also includes a few genes such as GADD34, a target of the stress activated transcription factor ATF4. Most of the transcript changes seen during the heat shock response were HSF1 independent as of the 308 genes of which the transcript levels changed at least two fold in HEK293 cells, only 30 are regulated by HSF1. The non-HSF1 dependent stress response is largely cell specific (Table 3B). A marker for a failing heat shock response in ageing cells will need to be sought among the common HSF1 regulated genes.

Table 3C Genes of which the transcripts are heat shock induced in both HEK293 and HeLa cells.

Gene name	Acc. Nr.	Gene name	Acc. Nr.
inhibited both K80Q and siRNA		**inhibited K80Q only**	
BAG3	NM_004281	ATF3	NM_001674
CRYAB	NM_001885	CYR61	NM_001554
DNAJB1	NM_006145	HSPA1A	NM_005345
HSPA6	NM_002155	JUN	NM_002228
HSPB1	NM_001540		
HSPH1	NM_006644		
SERPINH1	NM_001235		
inhibited siRNA only		**inhibited neither K80Q nor siRNA**	
ADM	NM_001124	DUSP5	NM_004419
RGS2	NM_002923	GADD45B	NM_015675
TXNIP	NM_006472	PPP1R15A	NM_014330
		TUFT1	NM_020127

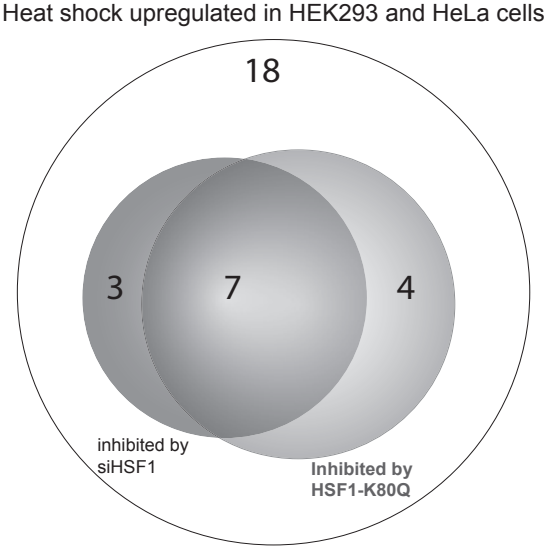


Figure 5 Schematic view of the heat shock induced transcriptome changes common to HEK293-HSF1-K80Q and siHSF1 HeLa cells.

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protein or amino acid induces C/EBPbeta synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription. Biochem J 2008, 410(3):473-484.
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Supplemental tables

Supplemental table 1 Oligonucleotides that were used to generate recombinant DNA constructs.

	Oligo name	Oligo sequence (5' →3')
Cloning	HSF1 sil. mut. (HSF1 WT)	cagaaagtcgtcaacaagcttatccagttcctgatctcactg
	HSF1-K80Q	catgtatggcttccggcaagtggtccacatcgagc
ChIP	HSPA6 ChIP fwd	ggaaggtgcgggaaggttcg
	HSPA6 ChIP rev	ttcttgtcggatgctgga
	BAG3 ChIP fwd	gattatagccgatgactcagggcg
	BAG3 ChIP rev	agtgtctggaaatagcctcc
	HSPH1 ChIP fwd	gaggcaggtttgagccaatg
	HSPH1 ChIP rev	cacttcctcagccttatgtatc
	HSPA1A ChIP fwd	ctctggagagttctgagcag
	HSPA1A ChIP rev	tataagtcgtcacggagacc
EMSA	HSE_EMSA_up	aacgagaatcttcgagaatggct
	HSE_EMSA_low	agccattctcgaagattctcgtt
QPCR	GAPDH_QPCR_fwd	gcagctgaaagaagcccaagt
	GAPDH_QPCR_rev	tgtcttccatgccaattgca
	HSPA1A_QPCR_fwd	ccgagaaggacgagtttgag
	HSPA1A_QPCR_rev	acaaaaacagcaatcttggaaagg
	HSPA6_QPCR_fwd	cagagatgaactttccctcc
	HSPA6_QPCR_rev	gaagcagaagaggatgaacc
	HSPA1B_QPCR_fwd	cagctctttgctgcttcac
	HSPA1B_QPCR_rev	cttacagtatcaacattaaatgc
	HSPA2_QPCR_fwd	actcaagtcagcgtaaacct
	HSPA2_QPCR_rev	aatagatctcgtacttggcac
	DNAJB1_QPCR_fwd	ttccccagacatcaagaacc
	DNAJB1_QPCR_rev	accctctcatggtccacaac
	HSP90_QPCR_fwd	gttggtcctgtgcggtcact
	HSP90_QPCR_rev	tgggcaatttctgcctgaa
	HSPB1_QPCR_fwd	cgcgctcagccggcaactc
	HSPB1_QPCR_rev	agccatgctcgtcctgccgc
	PCK2_QPCR_fwd	gcagcagaacacaaagggaag
	PCK2_QPCR_rev	tagtgcccgaagttgtagcc
	SGK_QPCR_fwd	cctgggagctgtcttgtatgag
	SGK_QPCR_rev	aggtgtcttgcggaatttgtaa
	ATF3_QPCR_fwd	tgccgaaacaagaagaagg
	ATF3_QPCR_rev	ttagctctgcaatgttccttc

	Oligo name	Oligo sequence (5' → 3')
QPCR	RGS2_QPCR_fwd	aagattggaagacccgtttgag
	RGS2_QPCR_rev	gcaagaccatatttgctggct
	GADD45B_QPCR_fwd	gacctgcattgtctcctggtc
	GADD45B_QPCR_rev	cagcgttcctgaagagagatgta
	GADD34_QPCR_fwd	cgcttctggcagaccgaa
	GADD34_QPCR_rev	gtagcctgatggggtgcttg
	BAG3_QPCR_fwd	ctccattccggtgatacacga
	BAG3_QPCR_rev	tggtgggtctggtactccc
	HSPH1_QPCR_fwd	aggagttccatatccagaa
	HSPH1_QCR_rev	cagctcaacattcaccac

Supplemental table 2 transcriptome changes in non-stressed (NS) cells and cells heat shocked and allowed to recover for 6 hrs (HS 6).

Gene name	SystematicName	ave K80Q+dox NS/pcDNA NS	sd K80Q+dox/pcDNA	ave HSF1 WT +dox NS/pcDNA5 NS	sd HSF1 WT +dox NS/pcDNA NS	ave K80Q+dox NS/HSF1 WT +dox NS (calculated)	sd K80Q+dox NS/HSF1 WT +dox NS (calculated)	ave pcDNA HS 6/pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS
increase in transcript level HSF1-K80Q NS relative to HSF1 WT NS											
ASNS	NM_001673	2,24	0,25	1,12	0,04	**1,99**	**0,29**	0,76	0,00	0,95	0,05
CTH	NM_001902	2,37	0,14	1,16	0,11	**2,04**	**0,07**	1,38	0,08	1,38	0,16
JUB	NM_032876	2,05	0,15	0,98	0,06	**2,09**	**0,02**	1,10	0,13	1,13	0,05
NUPR1	NM_001042483	1,40	0,18	0,59	0,03	**2,38**	**0,44**	1,01	0,06	1,38	0,30
PCK2	NM_004563	2,31	0,28	1,07	0,11	**2,18**	**0,44**	0,85	0,09	0,97	0,13
RNF125	NM_017831	1,98	0,09	1,01	0,55	**2,32**	**1,34**	0,88	0,05	0,95	0,05
SEPP1	NM_005410	37,02	6,34	13,29	1,72	**2,78**	**0,12**	0,88	0,37	0,96	0,04
SLC7A11	NM_014331	2,33	0,10	1,11	0,09	**2,10**	**0,07**	0,72	0,04	0,62	0,01
STC2	NM_003714	2,43	0,49	1,07	0,22	**2,26**	**0,16**	1,89	0,97	1,72	1,18
TPK1	NM_022445	2,94	0,37	1,27	0,21	**2,32**	**0,09**	1,16	0,24	0,98	0,01
WDR13	NM_017883	2,13	0,00	1,19	0,08	**1,79**	**0,12**	0,99	0,03	0,93	0,06
decrease in transcript level HSF1-K80Q NS relative to HSF1 WT NS											
BFSP1	NM_001195	0,36	0,01	1,48	0,16	**0,24**	**0,02**	1,06	0,05	1,10	0,08
C20orf201	NM_001007125	0,32	0,00	0,79	0,04	**0,41**	**0,02**	1,37	0,07	1,36	0,08
CGA	NM_000735	0,20	0,04	0,82	0,10	**0,24**	**0,03**	1,13	0,09	1,15	0,26
CRLF1	NM_004750	0,48	0,04	0,83	0,12	**0,58**	**0,12**	1,10	0,01	1,18	0,07
FERMT1	NM_017671	0,23	0,00	0,53	0,04	**0,43**	**0,04**	0,81	0,06	0,90	0,19
KRT222	NM_152349	0,46	0,06	0,97	0,08	**0,47**	**0,02**	0,81	0,08	0,87	0,07
LOC100292909	XM_002345507	0,50	0,03	1,13	0,19	**0,45**	**0,05**	0,48	0,14	0,51	0,02
LY6K	NM_017527	0,21	0,02	0,45	0,08	**0,47**	**0,13**	2,06	0,10	1,94	0,57
NPTX2	NM_002523	0,34	0,01	0,68	0,04	**0,50**	**0,05**	1,19	0,02	2,18	0,07
PLEKHG4	NM_015432	0,50	0,02	1,03	0,11	**0,49**	**0,04**	0,79	0,02	0,80	0,08
PPAPDC1A	NM_001030059	0,41	0,01	0,88	0,01	**0,46**	**0,02**	1,10	0,06	0,99	0,10
RAI2	NM_021785	0,39	0,03	1,61	0,12	**0,24**	**0,00**	0,62	0,04	0,90	0,13
SLC16A5	NM_004695	0,11	0,00	0,24	0,02	**0,44**	**0,03**	0,64	0,00	0,81	0,23
SYTL5	NM_001163335	0,18	0,01	0,37	0,02	**0,49**	**0,04**	1,02	0,06	0,98	0,02
UBE2C	NM_181803	0,50	0,00	1,09	0,02	**0,46**	**0,01**	1,21	0,04	1,16	0,02
VCX2	NM_016378	0,28	0,12	0,65	0,29	**0,43**	**0,01**	11,14	0,71	35,82	29,77
ZNF334	NM_199441	0,27	0,02	0,80	0,00	**0,33**	**0,03**	0,70	0,05	0,77	0,10

Supplemental table 3 transcriptome changes in cells 6 hours recovered from heat shock (HS 6) relative to non stressed (NS).

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
inhibited HSF1-K80Q						
HSPA1A	NM_005345	13,67	2,68	1,16	0,06	**11,8**
CRYAB	NM_001885	24,34	1,53	2,36	1,30	**10,3**
DNAJB1	NM_006145	32,62	2,54	3,41	0,73	**9,6**
HSPH1	NM_006644	9,81	1,69	1,89	0,09	**5,2**
CCDC121	NM_024584	4,66	0,49	1,26	0,11	**3,7**
USPL1	NM_005800	6,77	0,70	1,85	0,17	**3,7**
DHDH	NM_014475	5,69	2,09	1,62	0,34	**3,5**
DNAJB4	NM_007034	5,45	0,37	1,65	0,15	**3,3**
NXT2	NM_018698	5,48	0,76	1,67	0,10	**3,3**
ZFAND2A	NM_182491	5,74	0,66	1,76	0,34	**3,3**
C17orf67	ENST00000397861	2,89	0,24	0,92	0,03	**3,2**
BAG3	NM_004281	6,68	1,05	2,24	0,04	**3,0**
LOC387763	NM_001145033	2,80	0,27	0,94	0,20	**3,0**
DUSP1	NM_004417	11,85	0,86	4,11	0,12	**2,9**
UBB	NM_018955	3,29	0,68	1,23	0,18	**2,7**
LOC100130288	BC043212	2,05	0,02	0,84	0,03	**2,4**
LOC100133337	XM_002343495	2,27	0,15	0,97	0,08	**2,3**
MRPL18	NM_014161	2,45	0,24	1,06	0,04	**2,3**
ATF3	NM_001674	3,69	0,51	1,61	0,20	**2,3**
USP51	NM_201286	2,32	0,31	1,02	0,08	**2,3**
JUN	NM_002228	3,99	0,66	1,76	0,05	**2,3**
HSPA6	NM_002155	61,92	4,89	27,42	14,31	**2,3**
CACYBP	NM_014412	2,61	1,20	1,16	0,24	**2,2**
DEDD2	NM_133328	2,78	0,33	1,32	0,02	**2,1**
SNORD3D/BU532663	BU532663	4,31	0,52	2,09	1,11	**2,1**
UBC	NM_021009	2,81	0,30	1,39	0,12	**2,0**
C22orf43	NM_016449	3,20	0,31	1,59	0,07	**2,0**
HSP90AA1	NM_005348	2,77	0,61	1,38	0,13	**2,0**
SERPINH1	NM_001235	1,92	0,06	1,06	0,08	**1,8**
HSPB1	NM_001540	1,94	0,16	1,19	0,06	**1,6**
enhanced HSF1-K80Q						
DHRS2	NM_182908	3,71	0,31	7,68	3,20	**0,5**
NEFH	NM_021076	14,27	0,47	31,17	0,93	**0,5**
PLA2G4C	NM_003706	3,58	1,21	8,22	0,11	**0,4**
SGK1	NM_005627	11,68	1,15	30,22	4,99	**0,4**
HSPA2	NM_021979	2,71	0,69	7,05	2,62	**0,4**
VCX3A	NM_016379	10,16	0,40	27,54	20,26	**0,4**
OVGP1	NM_002557	2,74	0,15	7,48	1,42	**0,4**
VCX2	NM_016378	11,14	0,71	35,82	29,77	**0,3**
VCX	NM_013452	9,70	1,21	34,35	23,14	**0,3**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
ENST00000327934	ENST00000327934	2,72	0,31	1,40	0,09	**1,9**
CYR61	NM_001554	7,82	1,08	4,02	0,70	**1,9**
HSP90AB1	NM_007355	2,70	0,31	1,41	0,08	**1,9**
EGR1	NM_001964	232,11	72,57	121,01	24,99	**1,9**
DNAJB6	NM_058246	2,19	0,82	1,16	0,09	**1,9**
CLU	NM_203339	2,26	0,28	1,21	0,06	**1,9**
NR4A3	NM_173198	6,75	0,23	3,62	0,42	**1,9**
SNHG7	NR_003672	3,80	0,23	2,07	0,23	**1,8**
DDIT4	NM_019058	2,35	0,27	1,29	0,15	**1,8**
KLF6	NM_001300	2,14	0,06	1,19	0,02	**1,8**
NR4A1	NM_002135	6,30	0,95	3,50	0,35	**1,8**
EIF2AK3	NM_004836	2,33	0,11	1,30	0,35	**1,8**
LOC399851	AY129010	3,43	0,12	1,92	0,11	**1,8**
LOC646626	XM_942822	3,50	0,80	1,97	0,26	**1,8**
FOSB	NM_006732	44,68	6,34	25,66	5,88	**1,7**
ZNF10	NM_015394	3,75	0,61	2,18	0,32	**1,7**
FOS	NM_005252	70,19	4,29	41,08	4,19	**1,7**
SNORA24	BM926530	0,24	0,02	0,14	0,03	**1,7**
KCNJ2	NM_000891	2,62	0,43	1,55	0,31	**1,7**
LOC100289949	XM_002346836	3,18	0,30	1,90	0,34	**1,7**
LOC645955	XM_933296	2,26	0,21	1,35	0,10	**1,7**
MAFB	NM_005461	4,18	0,34	2,50	0,29	**1,7**
HMOX1	NM_002133	2,58	0,11	1,57	0,48	**1,6**
JMJD6	NM_015167	2,56	0,04	1,56	0,03	**1,6**
DUSP5	NM_004419	4,32	0,13	2,65	0,21	**1,6**
EIF1AD	NM_032325	2,32	1,86	1,44	0,87	**1,6**
IER2	NM_004907	5,19	0,26	3,24	0,23	**1,6**
RGS2	NM_002923	19,77	1,59	12,36	0,66	**1,6**
CHORDC1	NM_012124	2,08	0,39	1,33	0,01	**1,6**
TXNIP	NM_006472	4,38	1,10	2,81	0,75	**1,6**
OTP	NM_032109	2,13	0,34	1,41	0,15	**1,5**
FLJ23152	XM_001725362	2,91	0,12	1,97	0,06	**1,5**
ZNF436	NM_001077195	2,53	0,02	1,73	0,28	**1,5**
MXD1	NM_002357	2,29	0,42	1,58	0,37	**1,5**
KDM2A	NM_012308	2,60	0,18	1,79	0,21	**1,4**
CHAC1	NM_024111	2,84	0,01	1,99	0,71	**1,4**
CAMTA2	NM_015099	2,20	0,07	1,55	0,03	**1,4**
CTGF	NM_001901	6,20	1,87	4,45	0,11	**1,4**
ZC3H12A	NM_025079	4,82	0,02	3,46	0,18	**1,4**
GEM	NM_005261	5,07	0,99	3,66	0,87	**1,4**
C10orf10	NM_007021	6,20	0,48	4,48	1,09	**1,4**
SLC6A13 ENST00000436453	ENST00000436453	7,29	2,40	5,33	1,15	**1,4**
HERPUD1	NM_014685	4,67	0,25	3,45	0,94	**1,4**
BTN2A3	NR_027795	2,91	0,19	2,17	0,17	**1,3**
LOC100293390	XM_002346092	12,23	1,54	9,16	1,35	**1,3**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
ID2	NM_002166	2,09	0,11	1,57	0,08	**1,3**
TAC1	NM_003182	2,56	0,11	1,97	0,16	**1,3**
KIAA1683	NM_025249	2,50	0,71	1,93	0,05	**1,3**
ENST00000342294	ENST00000342294	2,60	0,67	2,00	0,15	**1,3**
KLF11	NM_003597	3,17	0,15	2,47	0,51	**1,3**
JUNB	NM_002229	3,68	1,08	2,87	1,07	**1,3**
ENST00000420072	ENST00000420072	0,50	0,02	0,39	0,01	**1,3**
DUSP8	NM_004420	2,72	1,43	2,16	0,82	**1,3**
KLF4	NM_004235	3,42	0,27	2,72	0,57	**1,3**
ARC	NM_015193	8,71	0,43	7,02	0,60	**1,2**
C14orf128	NR_027263	0,38	0,04	0,31	0,05	**1,2**
GADD45G	NM_006705	14,73	3,55	11,95	1,69	**1,2**
ENST00000454337	ENST00000454337	0,28	0,06	0,23	0,01	**1,2**
ENST00000392994	ENST00000392994	0,34	0,06	0,28	0,02	**1,2**
XM_002345305	XM_002345305	0,48	0,07	0,41	0,07	**1,2**
CX783461	CX783461	0,30	0,02	0,25	0,05	**1,2**
CRYGS	NM_017541	2,37	0,40	2,02	0,29	**1,2**
WEE1	NM_003390	2,89	0,19	2,46	0,12	**1,2**
SCARNA17	NR_003003	0,41	0,03	0,36	0,01	**1,2**
ING1	NM_198219	2,14	0,50	1,85	0,45	**1,2**
PPP1R15A	NM_014330	7,99	0,80	6,95	2,09	**1,1**
ETAA1	NM_019002	0,30	0,00	0,27	0,06	**1,1**
FLRT3	NM_198391	2,33	0,12	2,03	0,40	**1,1**
ENST00000451472	ENST00000451472	0,44	0,00	0,39	0,02	**1,1**
MGC16384	NR_026666	0,18	0,05	0,16	0,01	**1,1**
NPPC	NM_024409	2,05	0,49	1,81	0,50	**1,1**
TMEM60	NM_032936	0,49	0,05	0,43	0,05	**1,1**
FUT10	NM_032664	0,38	0,04	0,34	0,03	**1,1**
ENST00000414725	ENST00000414725	0,24	0,05	0,21	0,09	**1,1**
ENST00000440429	ENST00000440429	0,45	0,03	0,40	0,07	**1,1**
IER5L	NM_203434	5,95	0,50	5,34	1,05	**1,1**
FLJ44253	AK126241	0,44	0,13	0,40	0,07	**1,1**
MAT2A	NM_005911	0,35	0,04	0,32	0,02	**1,1**
DDX47	NM_016355	0,35	0,02	0,32	0,07	**1,1**
ZNF17	NM_006959	0,43	0,08	0,39	0,07	**1,1**
C19orf30	NR_027148	3,04	0,49	2,77	1,29	**1,1**
CLDN5	NM_003277	2,15	0,20	1,97	0,10	**1,1**
XIST	NR_001564	0,47	0,09	0,44	0,04	**1,1**
LEAP2	NM_052971	2,54	0,59	2,34	0,10	**1,1**
ZNF594	NM_032530	0,48	0,05	0,44	0,03	**1,1**
GADD45B	NM_015675	31,97	12,65	29,94	14,04	**1,1**
ZNF616	BC032805	0,44	0,03	0,41	0,07	**1,1**
LY6K	NM_017527	2,06	0,10	1,94	0,57	**1,1**
C20orf111	NM_016470	2,05	0,04	1,95	0,06	**1,1**
KIAA1949	NM_133471	2,31	0,25	2,20	0,18	**1,0**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
PRDX1	NM_002574	0,45	0,03	0,43	0,08	**1,0**
ENST00000392875	ENST00000392875	0,46	0,09	0,45	0,02	**1,0**
VEGFA	NM_001025370	2,31	1,29	2,23	0,68	**1,0**
LOC388630	XM_371250	0,43	0,03	0,42	0,03	**1,0**
ZBTB2	NM_020861	2,40	0,16	2,33	0,13	**1,0**
ENST00000431042	ENST00000431042	2,09	0,21	2,03	0,23	**1,0**
U72516	U72516	0,48	0,09	0,47	0,08	**1,0**
ZBTB26	ENST00000373656	0,45	0,02	0,44	0,04	**1,0**
LOC644354	XM_002343524	0,43	0,11	0,42	0,03	**1,0**
SFRS17A	NM_005088	2,34	0,14	2,30	0,24	**1,0**
KLF10	NM_005655	6,74	0,51	6,64	2,06	**1,0**
PRRT2	NM_145239	2,97	1,28	2,93	0,46	**1,0**
LOC100288418	XM_002342023	0,29	0,09	0,28	0,08	**1,0**
GJC2	NM_020435	2,49	0,45	2,47	0,86	**1,0**
PTX3	NM_002852	0,49	0,03	0,49	0,02	**1,0**
GEMIN6	NM_024775	0,26	0,01	0,26	0,03	**1,0**
ZNF526	NM_133444	0,41	0,01	0,41	0,02	**1,0**
BCDIN3D	NM_181708	0,38	0,00	0,38	0,05	**1,0**
DNAJB9	NM_012328	2,04	0,01	2,03	0,38	**1,0**
GIN1	NM_017676	0,44	0,04	0,44	0,13	**1,0**
C5orf58	NM_001102609	0,49	0,07	0,49	0,05	**1,0**
ENST00000452128	ENST00000452128	0,43	0,00	0,44	0,04	**1,0**
ZNF184	NM_007149	2,58	0,11	2,59	0,17	**1,0**
HDAC5	NM_001015053	2,29	1,73	2,31	1,85	**1,0**
FADD	NM_003824	0,43	0,05	0,43	0,07	**1,0**
LOC100288578	XR_078216	0,32	0,08	0,32	0,07	**1,0**
ZNF337	NM_015655	0,41	0,04	0,41	0,07	**1,0**
PARD3B	NM_205863	0,49	0,01	0,50	0,16	**1,0**
LOC100291981	XM_002346163	2,74	1,69	2,80	1,23	**1,0**
RWDD2A	NM_033411	0,47	0,01	0,48	0,08	**1,0**
RFPL3S	NR_001450	2,93	0,34	3,03	0,50	**1,0**
C7orf68	NM_013332	0,31	0,02	0,32	0,01	**1,0**
C3orf50	NR_021485	0,42	0,07	0,43	0,02	**1,0**
ACTA1	NM_001100	13,84	3,80	14,38	1,08	**1,0**
GPX8	NM_001008397	0,42	0,05	0,44	0,06	**1,0**
DGCR8	NM_022720	0,41	0,15	0,43	0,12	**1,0**
C17orf100	NM_001105520	0,43	0,02	0,45	0,00	**1,0**
ID4	NM_001546	0,43	0,01	0,45	0,01	**1,0**
LOC100288891	XM_002342041	2,22	0,33	2,32	0,22	**1,0**
RGS9BP	NM_207391	0,49	0,10	0,52	0,04	**1,0**
LOC100292909	XM_002345507	0,48	0,14	0,51	0,02	**1,0**
OXTR	NM_000916	0,46	0,04	0,49	0,01	**0,9**
FAM90A1	NM_018088	5,56	0,31	5,87	1,16	**0,9**
SIRT4	NM_012240	5,09	0,24	5,39	0,05	**0,9**
FAM46A	NM_017633	2,94	0,24	3,11	0,52	**0,9**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
C15orf39	NM_015492	0,38	0,04	0,40	0,03	**0,9**
ADM	NM_001124	6,19	0,97	6,58	1,03	**0,9**
CSTF1	NM_001324	0,40	0,02	0,42	0,09	**0,9**
C8orf48	NM_001007090	0,49	0,04	0,52	0,10	**0,9**
TRMT12	NM_017956	0,46	0,02	0,50	0,04	**0,9**
MED4	NM_014166	0,44	0,06	0,47	0,03	**0,9**
ZBTB45	NM_032792	0,39	0,01	0,42	0,05	**0,9**
TRIM69	NM_182985	2,16	0,03	2,32	0,70	**0,9**
LCMT2	NM_014793	0,36	0,01	0,39	0,09	**0,9**
ZNF792	NM_175872	0,50	0,01	0,54	0,03	**0,9**
MTERF	NM_006980	0,43	0,04	0,47	0,15	**0,9**
RRAD	NM_004165	4,27	0,29	4,63	1,22	**0,9**
ZNF767	NR_027788	0,47	0,00	0,51	0,04	**0,9**
BBS12	NM_152618	0,36	0,07	0,39	0,10	**0,9**
SMCR7	NM_139162	0,40	0,02	0,43	0,04	**0,9**
ZNF264	NM_003417	0,37	0,01	0,40	0,02	**0,9**
C5orf41	NM_153607	2,29	0,05	2,49	0,12	**0,9**
TIGD5	NM_032862	0,48	0,04	0,53	0,01	**0,9**
BCL11B	NM_138576	0,49	0,04	0,53	0,07	**0,9**
HSPA5	NM_005347	4,43	3,31	4,84	3,86	**0,9**
SERTAD3	NM_203344	2,84	0,56	3,10	0,63	**0,9**
LOC728485	XR_041662	0,50	0,04	0,55	0,05	**0,9**
FAM90A7	NM_001136572	5,73	0,27	6,30	1,03	**0,9**
DYRK3	NM_001004023	0,49	0,11	0,54	0,10	**0,9**
ENPP5	NM_021572	0,48	0,03	0,53	0,02	**0,9**
IRX5	NM_005853	2,13	0,13	2,35	0,49	**0,9**
ZSWIM1	NM_080603	0,50	0,04	0,55	0,06	**0,9**
TMEM177	NM_030577	0,33	0,00	0,36	0,04	**0,9**
MED11	NM_001001683	0,31	0,05	0,34	0,05	**0,9**
TMEM187	NM_003492	0,43	0,04	0,48	0,10	**0,9**
ZNF564	NM_144976	0,32	0,03	0,35	0,06	**0,9**
PLK2	NM_006622	3,52	0,12	3,90	0,17	**0,9**
XRCC2	NM_005431	0,40	0,03	0,44	0,02	**0,9**
SNRNP35	NM_022717	0,49	0,02	0,54	0,10	**0,9**
KLF5	NM_001730	2,42	0,15	2,69	0,31	**0,9**
CBX4	NM_003655	2,80	0,63	3,12	0,26	**0,9**
C1GALT1C1	NM_152692	0,38	0,02	0,42	0,05	**0,9**
PTRH2	NM_016077	0,48	0,02	0,54	0,07	**0,9**
LOC151878	BC014063	0,30	0,09	0,33	0,01	**0,9**
LOH3CR2A	NR_024065	2,11	0,73	2,36	0,16	**0,9**
KCNJ8	NM_004982	0,40	0,07	0,45	0,03	**0,9**
KIAA0754	NM_015038	0,39	0,06	0,44	0,05	**0,9**
EID2B	NM_152361	0,35	0,06	0,39	0,07	**0,9**
MCM9	NM_153255	0,31	0,05	0,34	0,03	**0,9**
CAMTA1	AY037153	2,64	1,36	2,99	1,67	**0,9**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
EZH1	NM_001991	0,45	0,04	0,52	0,05	**0,9**
ZNF485	NM_145312	0,48	0,07	0,54	0,09	**0,9**
LOC100270746	NR_026776	0,33	0,01	0,38	0,09	**0,9**
RNF43	NM_017763	0,39	0,00	0,45	0,06	**0,9**
ARL10	ENST00000310389	0,45	0,02	0,52	0,07	**0,9**
FBXL21	NM_012159	0,46	0,04	0,53	0,00	**0,9**
CCDC146	NM_020879	0,48	0,07	0,55	0,03	**0,9**
MED7	NM_004270	0,28	0,06	0,32	0,07	**0,9**
MFAP1	NM_005926	0,40	0,00	0,47	0,06	**0,9**
ENST00000369399	ENST00000369399	0,44	0,10	0,51	0,05	**0,9**
ZNF394	NM_032164	3,26	0,23	3,80	0,20	**0,9**
RSRC2	NM_198261	2,17	0,30	2,52	0,23	**0,9**
GEMIN4	NM_015721	0,32	0,02	0,38	0,06	**0,9**
PPP1R3E	NR_026862	0,42	0,03	0,49	0,03	**0,9**
ELK4	ENST00000357992	0,45	0,03	0,52	0,02	**0,9**
ALX1	NM_006982	0,42	0,08	0,49	0,12	**0,9**
WDR5B	NM_019069	0,30	0,01	0,35	0,09	**0,9**
ENST00000344859	ENST00000344859	0,30	0,04	0,35	0,04	**0,9**
C5orf53	NM_001007189	0,40	0,01	0,47	0,02	**0,8**
SMAD7	NM_005904	2,36	0,09	2,78	0,62	**0,8**
CROCCL1	NR_026752	0,44	0,05	0,52	0,05	**0,8**
TMEM121	NM_025268	0,34	0,01	0,40	0,05	**0,8**
IFIT1	NM_001548	0,45	0,02	0,53	0,05	**0,8**
FLJ36840	AK094159	0,40	0,12	0,48	0,03	**0,8**
C1orf156	NM_033418	0,38	0,01	0,46	0,02	**0,8**
NAT1	NM_000662	0,45	0,01	0,54	0,03	**0,8**
LOC401397	NR_024412	0,33	0,06	0,40	0,09	**0,8**
NPC1L1	NM_013389	4,88	0,56	5,90	0,28	**0,8**
SEMA3B	NM_004636	2,30	0,72	2,79	0,60	**0,8**
S100P	NM_005980	16,72	0,14	20,49	0,55	**0,8**
LOC100290355	XM_002347747	0,45	0,04	0,55	0,12	**0,8**
LOC390595	NM_001163692	0,32	0,05	0,39	0,07	**0,8**
LOC728705	BC034428	0,35	0,03	0,43	0,09	**0,8**
ZNF555	NM_152791	0,42	0,03	0,53	0,01	**0,8**
LOC100287911	XM_002342123	2,16	0,48	2,70	0,24	**0,8**
SNIP1	NM_024700	2,52	0,14	3,17	0,37	**0,8**
KLF2	NM_016270	2,07	0,39	2,61	1,16	**0,8**
HPS6	NM_024747	0,29	0,04	0,37	0,01	**0,8**
TUFT1	NM_020127	2,24	0,19	2,82	0,04	**0,8**
ASGR1	NM_001671	2,14	0,49	2,70	0,60	**0,8**
LYPD3	NM_014400	3,93	0,63	4,97	0,17	**0,8**
BRPF1	NM_001003694	2,00	0,41	2,54	0,61	**0,8**
LOC286272	AK093004	0,47	0,05	0,60	0,05	**0,8**
VCY	NM_004679	3,80	0,46	4,95	2,25	**0,8**
MED26	NM_004831	2,87	0,06	3,74	0,18	**0,8**

GeneName	SystematicName	ave pcDNA HS 6/ pcDNA NS	sd pcDNA HS 6/pcDNA NS	ave K80Q+dox HS 6/K80Q+dox NS	sd K80Q+dox HS 6/K80Q+dox NS	fold inhibition HSF1-K80Q
no change HSF1-K80Q						
CCDC87	NM_018219	0,33	0,04	0,43	0,09	**0,8**
DLL1	NM_005618	3,58	0,20	4,71	1,72	**0,8**
HIST1H2AC	ENST00000314088	2,36	0,28	3,11	0,31	**0,8**
PTGER4	NM_000958	3,29	0,40	4,35	0,99	**0,8**
TAC3	NM_013251	3,56	0,86	4,71	0,57	**0,8**
DISP1	NM_032890	0,48	0,06	0,63	0,01	**0,7**
MIR17HG	NR_027350	0,43	0,08	0,57	0,03	**0,7**
IER3	NM_003897	4,05	0,49	5,46	0,82	**0,7**
SERTAD1	NM_013376	5,65	0,01	7,64	0,25	**0,7**
TMPRSS5	NM_030770	0,44	0,02	0,60	0,11	**0,7**
C10orf110	NR_027709	4,68	2,27	6,40	1,94	**0,7**
FLJ10213	NM_018029	0,27	0,08	0,37	0,00	**0,7**
HBEGF	NM_001945	6,09	0,93	8,47	1,50	**0,7**
FRG2	NM_001005217	7,69	0,05	10,73	2,22	**0,7**
ENST00000335883	ENST00000335883	0,45	0,08	0,63	0,14	**0,7**
C1S	NM_001734	2,73	0,89	3,91	0,47	**0,7**
RPL13P5	NR_002803	8,60	2,61	12,39	1,15	**0,7**
C1orf162	NM_174896	4,01	0,84	5,84	0,37	**0,7**
TIGD3	NM_145719	3,12	0,19	4,58	0,41	**0,7**
FAM106A	NR_026809	0,30	0,02	0,45	0,00	**0,7**
RIMBP3	NM_015672	0,44	0,08	0,66	0,08	**0,7**
NXF1	NM_006362	2,40	0,29	3,63	0,00	**0,7**
C5orf27	NR_026936	3,38	0,07	5,13	2,31	**0,7**
ARL4D	NM_001661	2,48	0,35	3,82	0,76	**0,7**
LOC100130401	BC069659	0,22	0,10	0,34	0,11	**0,6**
ELP2P	NR_024120	3,11	0,01	4,91	0,20	**0,6**
ZFP36	NM_003407	8,44	0,05	13,34	2,24	**0,6**
C7orf53	NM_182597	3,80	0,42	6,13	1,99	**0,6**
SNAI1	NM_005985	27,62	1,55	45,06	2,29	**0,6**
ZNF572	NM_152412	0,31	0,08	0,52	0,01	**0,6**
AOC3	NM_003734	6,00	3,37	9,91	0,07	**0,6**
HOXD1	NM_024501	8,57	0,89	14,26	4,58	**0,6**
LOC80054	NR_026887	0,38	0,00	0,64	0,00	**0,6**
GOLGA6L6	NM_001145004	2,27	0,65	3,80	0,18	**0,6**
ENST00000417642 retained intron	ENST00000417642	0,40	0,03	0,68	0,01	**0,6**
KCNJ14	NM_170720	2,56	1,32	4,39	1,46	**0,6**
CCDC96	NM_153376	2,86	0,41	4,90	0,47	**0,6**
CCDC11	NM_145020	10,23	3,98	17,61	4,25	**0,6**
GFPT2	NM_005110	2,66	0,30	4,62	0,09	**0,6**
MYLK2	NM_033118	3,26	1,12	5,67	2,64	**0,6**
C1orf63	NM_020317	5,78	0,43	10,10	0,82	**0,6**
ACRC	NM_052957	3,57	1,03	6,41	0,62	**0,6**
CDKN1C	NM_000076	2,00	0,02	3,87	0,56	**0,5**
RASD1	NM_016084	129,09	22,99	256,48	10,63	**0,5**

Supplemental table 4A comparison of transcriptome changes in non-stressed siHSF1 treated HeLa cells or HSF1-K80Q HEK293 cells (NS).

Gene name	SystematicName	ave siHSF1 v siLUC TO	sd siHSF1 v siLUC TO	ave K80Q+dox NS/pcDNA NS	sd K80Q+dox/pcDNA	ave HSF1 WT +dox NS/pcDNA NS	sd HSF1 WT +dox NS/pcDNA NS	ave K80Q+dox NS/HSF1 WT +dox NS (calculated)	sd K80Q+dox NS/HSF1 WT +dox NS (calculated)
inhibited both HSF1-K80Q and siRNA HSF1									
CRLF1	NM_004750	**0,48**	0,07	0,48	0,04	**0,83**	**0,12**	**0,58**	0,12
inhibited HSF1-K80Q not siRNA HSF1									
CGA	NM_000735	**0,71**	0,65	0,20	0,04	0,82	0,10	**0,24**	0,03
BFSP1	NM_001195	**1,02**	0,24	0,36	0,01	1,48	0,16	**0,24**	0,02
RAI2	NM_021785	**1,02**	0,02	0,39	0,03	1,61	0,12	**0,24**	0,00
ZNF334	NM_199441	**1,09**	0,01	0,27	0,02	0,80	0,00	**0,33**	0,03
VCX2	NM_016378	**1,28**	0,17	0,28	0,12	0,65	0,29	**0,43**	0,01
SLC16A5	NM_004695	**0,84**	0,10	0,11	0,00	0,24	0,02	**0,44**	0,03
UBE2C	NM_181803	**1,03**	0,07	0,50	0,00	1,09	0,02	**0,46**	0,01
NPTX2	NM_002523	**1,02**	0,02	0,34	0,01	0,68	0,04	**0,50**	0,05

Supplemental table 4B comparison of transcriptome changes in heat shocked siHSF1 treated HeLa cells or HSF1-K80Q HEK293 cells (HS 6).

Gene name	Systematic-Name	ave T4 vT0	sd	ave siHSF1 v siLUC T4	sd	ave pcDNA HS 6/ pcDNA NS	sd	ave K80Q+dox HS 6/ K80Q+dox NS	sd	fold inhibition HSF1-K80Q	ave dnHSF1+dox HS 6/ dnHSF1+dox NS	sd	fold inhibition dnHSF1
inhibted both K80Q and siRNA													
CRYAB	NM_001885	2,24		**0,28**	0,09	24,34	1,53	2,36	1,30	**10,3**	3,62	0,19	6,7
BAG3	NM_004281	4,26		**0,37**	0,14	6,68	1,05	2,24	0,04	**3,0**	1,31	0,14	5,1
HSPA6	NM_002155	16,70		**0,39**	0,19	61,92	4,89	27,42	14,31	**2,3**	18,09	3,80	3,4
HSPH1	NM_006644	2,82	0,34	**0,47**	0,12	9,81	1,69	1,89	0,09	**5,2**	0,98	0,18	10,0
SERPINH1	NM_001235	2,12		**0,47**	0,03	1,92	0,06	1,06	0,08	**1,8**	0,31	0,00	6,2
DNAJB1	NM_006145	2,92	0,11	**0,51**	0,15	32,62	2,54	3,41	0,73	**9,6**	0,84	0,01	38,8
HSPB1	NM_001540	1,71		**0,54**	0,13	1,94	0,16	1,19	0,06	**1,6**	0,47	0,03	4,2
inhibted K80Q but not siRNA													
HSPA1A	NM_005345	1,98		**0,83**	0,17	13,67	2,68	1,16	0,06	**11,8**	1,04	0,09	13,2
ATF3	NM_001040619	2,41		**0,89**	0,15	3,69	0,51	1,61	0,20	**2,3**	2,53	0,36	1,5
JUN	NM_002228	2,00	0,41	**0,90**	0,19	3,99	0,66	1,76	0,05	**2,3**	1,41	0,24	2,8
CYR61	NM_001554	2,01	0,31	**0,93**	0,11	7,82	1,08	4,02	0,70	**1,9**	5,00	0,77	1,6
inhibted siRNA but not K80Q													
TXNIP	NM_006472	2,59	0,59	**0,34**	0,06	4,38	1,10	2,81	0,75	**1,6**	4,62	1,41	0,9
ADM	NM_001124	2,26		**0,48**	0,00	6,19	0,97	6,58	1,03	**0,9**	3,59	0,62	1,7
RGS2	NM_002923	2,04		**0,54**	0,01	19,77	1,59	12,36	0,66	**1,6**	13,68	2,14	1,4
not inhibited siRNA or K80Q													
TUFT1	NM_020127	2,93		**0,97**	0,26	2,24	0,19	2,82	0,04	**0,8**	2,04	0,02	1,1
PPP1R15A	NM_014330	2,64	0,15	**0,61**	0,04	7,99	0,80	6,95	2,09	**1,1**	6,11	0,05	1,3
DUSP5	NM_004419	2,58		**0,63**	0,16	4,32	0,13	2,65	0,21	**1,6**	1,79	0,39	2,4
GADD45B	NM_015675	2,05	0,63	**0,73**	0,22	31,97	12,65	29,94	14,04	**1,1**	13,37	4,41	2,4

Gene name	Systematic-Name	ave T4 vT0	sd	ave siHSF1 v siLUC T4	sd	ave pcDNA HS 6/ pcDNA NS	sd	ave K80Q+dox HS 6/ K80Q+dox NS	sd	fold inhibition HSF1-K80Q	ave dnHSF1+dox HS 6/ dnHSF1+dox NS	sd	fold inhibition dnHSF1
lower in both HEK-HSF1-K80Q and HeLa cells after heat stress													
ZNF264	NM_003417	**0,44**		1,57	0,59	**0,37**	0,01	0,4	0,02	0,9	0,39	0,06	0,9
heat shock induced HEK293 but not heat shock induced in HeLa cells													
K80Q inhibited													
CCDC121	NM_024584	**1,19**		0,77	0,16	**4,66**	0,49	1,26	0,11	**3,7**	1,91	0,14	2,4
DNAJB4	NM_007034	**0,92**	0,14	0,83	0,19	**5,45**	0,37	1,65	0,15	**3,3**	1,18	0,05	4,6
NXT2	NM_018698	**1,03**	0,32	0,98	0,10	**5,48**	0,76	1,67	0,10	**3,3**	1,03	0,15	5,3
DUSP1	NM_004417	**0,95**	0,10	1,03	0,18	**11,85**	0,86	4,11	0,12	**2,9**	4,42	1,06	2,7
UBB	NM_018955	**1,29**	0,22	0,90	0,12	**3,29**	0,68	1,23	0,18	**2,7**	1,03	0,19	3,2
MRPL18	NM_014161	**1,76**		0,68	0,10	**2,45**	0,24	1,06	0,04	**2,3**	0,80	0,01	3,1
CACYBP	NM_014412	**1,42**	0,19	0,74	0,11	**2,61**	1,20	1,16	0,24	**2,2**	0,82	0,25	3,2
UBC	NM_021009	**0,89**	0,06	1,07	0,05	**2,81**	0,30	1,39	0,12	**2,0**	1,65	0,25	1,7
C22orf43	NM_016449	**1,08**		0,99	0,09	**3,20**	0,31	1,59	0,07	**2,0**	2,04	0,19	1,6
K80Q enhanced													
VCX2	NM_016378	**1,35**		0,98	0,30	**11,14**	0,71	35,82	29,77	**0,3**	5,85	0,30	1,9
OVGP1	NM_002557	**1,19**		0,98	0,05	**2,74**	0,15	7,48	1,42	**0,4**	2,80	0,07	1,0
HSPA2	NM_021979	**0,86**		1,28	0,53	**2,71**	0,69	7,05	2,62	**0,4**	5,49	0,07	0,5
SGK1	NM_005627	**1,09**		1,01	0,07	**11,68**	1,15	30,22	4,99	**0,4**	13,53	0,88	0,9
PLA2G4C	NM_003706	**1,14**		0,98	0,03	**3,58**	1,21	8,22	0,11	**0,4**	3,08	0,22	1,2
NEFH	NM_021076	**1,04**	0,10	1,20	0,37	**14,27**	0,47	31,17	0,93	**0,5**	12,19	0,07	1,2
DHRS2	NM_182908	**1,43**	0,11	0,69	0,07	**3,71**	0,31	7,68	3,20	**0,5**	2,17	0,09	1,7

Gene name	Systematic-Name	ave T4 vT0	sd	ave siHSF1 v siLUC T4	sd	ave pcDNA HS 6/ pcDNA NS	sd	ave K80Q+dox HS 6/ K80Q+dox NS	sd	fold inhibition HSF1-K80Q	ave dnHSF1+dox HS 6/ dnHSF1+dox NS	sd	fold inhibition dnHSF1
no effect K80Q													
CDKN1C	NM_000076	1,23	0,45	0,75	0,20	2,00	0,02	3,87	0,56	**0,5**	8,38	0,09	0,2
C1orf63	NM_020317	0,39	0,14	1,48	0,19	5,78	0,43	10,10	0,82	**0,6**	8,78	0,41	0,7
GFPT2	NM_005110	0,87		0,52	0,26	2,66	0,30	4,62	0,09	**0,6**	2,48	0,20	1,1
KCNJ14	NM_170720	1,06		1,02	0,03	2,56	1,32	4,39	1,46	**0,6**	1,41	0,15	1,8
HOXD1	NM_024501	0,97	0,11	1,04	0,04	8,57	0,89	14,26	4,58	**0,6**	18,64	3,86	0,5
AOC3	NM_003734	0,87		1,10	0,10	6,00	3,37	9,91	0,07	**0,6**	1,73	0,61	3,5
SNAI1	NM_005985	1,13		0,89	0,01	27,62	1,55	45,06	2,29	**0,6**	28,57	5,12	1,0
ZFP36	NM_003407	1,48		0,64	0,01	8,44	0,05	13,34	2,24	**0,6**	9,53	0,93	0,9
NXF1	NM_006362	0,69		1,33	0,02	2,40	0,29	3,63	0,00	**0,7**	2,29	0,32	1,0
C1S	NM_001734	0,87		0,62	0,05	2,73	0,89	3,91	0,47	**0,7**	3,26	0,25	0,8
HBEGF	NM_001945	1,33	0,09	0,92	0,07	6,09	0,93	8,47	1,50	**0,7**	6,85	1,77	0,9
C10orf110	NR_027709	0,75		1,49	0,09	4,68	2,27	6,40	1,94	**0,7**	1,26	0,37	3,7
IER3	NM_003897	1,33		1,11	0,07	4,05	0,49	5,46	0,82	**0,7**	3,51	0,68	1,2
TAC3	NM_013251	1,08		1,01	0,03	3,56	0,86	4,71	0,57	**0,8**	3,18	1,17	1,1
PTGER4	NM_000958	1,49	0,48	0,95	0,16	3,29	0,40	4,35	0,99	**0,8**	4,79	0,58	0,7
HIST1H2AC	ENST00000314088	1,56		0,44	0,08	2,36	0,28	3,11	0,31	**0,8**	3,78	1,09	0,6
BRPF1	NM_001003694	1,04		1,03	0,01	2,00	0,41	2,54	0,61	**0,8**	1,39	0,03	1,4
ASGR1	NM_001671	1,29		0,93	0,15	2,14	0,49	2,70	0,60	**0,8**	1,76	0,36	1,2
KLF2	NM_016270	0,91		0,91	0,27	2,07	0,39	2,61	1,16	**0,8**	2,49	0,18	0,8
SNIP1	NM_024700	1,00		1,05	0,05	2,52	0,14	3,17	0,37	**0,8**	3,00	0,21	0,8
S100P	NM_005980	1,65		0,54	0,05	16,72	0,14	20,49	0,55	**0,8**	20,70	1,91	0,8
SEMA3B	NM_004636	1,20	0,06	0,79	0,22	2,30	0,72	2,79	0,60	**0,8**	2,69	0,77	0,9
NPC1L1	NM_013389	1,18		1,00	0,17	4,88	0,56	5,90	0,28	**0,8**	2,56	0,34	1,9
SMAD7	NM_005904	1,27		0,97	0,12	2,36	0,09	2,78	0,62	**0,8**	1,67	0,10	1,4
RSRC2	NM_198261	0,70	0,11	1,69	0,24	2,17	0,30	2,52	0,23	**0,9**	1,07	0,14	2,0
ZNF394	NM_032164	1,13		1,02	0,17	3,26	0,23	3,80	0,20	**0,9**	1,80	0,12	1,8
CAMTA1	AY037153	1,13		1,03	0,12	2,64	1,36	2,99	1,67	**0,9**	2,46	1,23	1,1
LOH3CR2A	NR_024065	0,94		1,04	0,05	2,11	0,73	2,36	0,16	**0,9**	4,81	0,63	0,4
CBX4	NM_003655	1,94		0,66	0,08	2,80	0,63	3,12	0,26	**0,9**	2,50	0,13	1,1
KLF5	NM_001730	0,86	0,06	2,00	0,29	2,42	0,15	2,69	0,31	**0,9**	2,00	0,43	1,2
PLK2	NM_006622	1,43		1,34	0,10	3,52	0,12	3,90	0,17	**0,9**	3,81	0,41	0,9
IRX5	NM_005853	0,77		1,00	0,35	2,13	0,13	2,35	0,49	**0,9**	2,36	0,12	0,9
SERTAD3	NM_203344	0,83		1,00	0,16	2,84	0,56	3,10	0,63	**0,9**	1,91	0,31	1,5
HSPA5	NM_005347	1,39		0,89	0,27	4,43	3,31	4,84	3,86	**0,9**	2,77	1,96	1,6
RRAD	NM_004165	1,44	0,09	0,97	0,12	4,27	0,29	4,63	1,22	**0,9**	4,74	0,90	0,9
CSTF1	NM_001324	0,81	0,02	1,22	0,16	0,40	0,02	0,42	0,09	**0,9**	0,37	0,07	1,1
FAM46A	NM_017633	0,57		1,34	0,40	2,94	0,24	3,11	0,52	**0,9**	3,51	0,41	0,8
SIRT4	NM_012240	1,01	0,02	1,01	0,01	5,09	0,24	5,39	0,05	**0,9**	2,64	0,32	1,9
ACTA1	NM_001100	1,03		1,01	0,03	13,84	3,80	14,38	1,08	**1,0**	5,55	1,01	2,5
RFPL3S	NR_001450	0,96		0,94	0,01	2,93	0,34	3,03	0,50	**1,0**	3,10	0,67	0,9
HDAC5	NM_001015053	1,24		0,89	0,37	2,29	1,73	2,31	1,85	**1,0**	2,45	1,34	0,9
ZNF184	NM_007149	0,69		0,92	0,19	2,58	0,11	2,59	0,17	**1,0**	2,61	0,06	1,0
DNAJB9	NM_012328	0,79	0,01	0,83	0,17	2,04	0,01	2,03	0,38	**1,0**	1,27	0,03	1,6

Gene name	Systematic-Name	ave T4 vT0	sd	ave siHSF1 v siLUC T4	sd	ave pcDNA HS 6/ pcDNA NS	sd	ave K80Q+dox HS 6/ K80Q+dox NS	sd	fold inhibi-tion HSF1-K80Q	ave dnHSF1+dox HS 6/ dnHSF1+dox NS	sd	fold inhibition dnHSF1
no effect K80Q													
GJC2	NM_020435	1,13	0,01	0,97	0,10	2,49	0,45	2,47	0,86	**1,0**	1,26	0,13	2,0
KLF10	NM_005655	1,32		0,98	0,06	6,74	0,51	6,64	2,06	**1,0**	7,51	1,40	0,9
C20orf111	NM_016470	1,04	0,07	0,96	0,11	2,05	0,04	1,95	0,06	**1,1**	1,41	0,13	1,5
CLDN5	NM_003277	1,18		0,79	0,02	2,15	0,20	1,97	0,10	**1,1**	3,89	0,11	0,6
NPPC	NM_024409	1,28		0,87	0,08	2,05	0,49	1,81	0,50	**1,1**	1,60	0,32	1,3
FLRT3	NM_198391	0,85		1,10	0,17	2,33	0,12	2,03	0,40	**1,1**	1,83	0,41	1,3
ING1	NM_198219	0,70	0,08	1,28	0,07	2,14	0,50	1,85	0,45	**1,2**	2,36	0,72	0,9
WEE1	NM_003390	0,97	0,07	0,92	0,19	2,89	0,19	2,46	0,12	**1,2**	1,81	0,29	1,6
GADD45G	NM_006705	1,38		0,96	0,12	14,73	3,55	11,95	1,69	**1,2**	8,64	1,14	1,7
ARC	NM_015193	1,22		0,92	0,11	8,71	0,43	7,02	0,60	**1,2**	8,19	0,73	1,1
KLF4	NM_004235	1,62	0,14	0,68	0,09	3,42	0,27	2,72	0,57	**1,3**	3,12	0,46	1,1
DUSP8	NM_004420	1,18		1,02	0,02	2,72	1,43	2,16	0,82	**1,3**	1,77	0,47	1,5
JUNB	NM_002229	1,58		0,85	0,00	3,68	1,08	2,87	1,07	**1,3**	3,66	0,65	1,0
KLF11	NM_003597	1,16		0,96	0,47	3,17	0,15	2,47	0,51	**1,3**	3,42	0,63	0,9
TAC1	NM_003182	1,04		0,98	0,01	2,56	0,11	1,97	0,16	**1,3**	3,13	0,40	0,8
ID2	NM_002166	0,46		0,63	0,56	2,09	0,11	1,57	0,08	**1,3**	2,25	0,07	0,9
BTN2A3	NR_027795	1,13		0,91	0,15	2,91	0,19	2,17	0,17	**1,3**	1,06	0,23	2,8
HERPUD1	NM_014685	1,72		0,92	0,20	4,67	0,25	3,45	0,94	**1,4**	2,35	0,73	2,0
C10orf10	NM_007021	1,29	0,16	0,93	0,12	6,20	0,48	4,48	1,09	**1,4**	2,68	0,46	2,3
GEM	NM_005261	1,14		0,88	0,22	5,07	0,99	3,66	0,87	**1,4**	5,88	0,28	0,9
ZC3H12A	NM_025079	1,61		0,66	0,02	4,82	0,02	3,46	0,18	**1,4**	3,18	0,59	1,5
CAMTA2	NM_015099	1,53		1,13	0,28	2,20	0,07	1,55	0,03	**1,4**	1,55	0,46	1,4
KDM2A	NM_012308	0,76	0,18	1,25	0,35	2,60	0,18	1,79	0,21	**1,4**	1,04	0,00	2,5
MXD1	NM_002357	1,24		1,00	0,07	2,29	0,42	1,58	0,37	**1,5**	1,28	0,24	1,8
CHORDC1	NM_012124	1,05		1,06	0,07	2,08	0,39	1,33	0,01	**1,6**	0,65	0,05	3,2
IER2	NM_004907	1,25		0,93	0,06	5,19	0,26	3,24	0,23	**1,6**	4,23	0,42	1,2
EIF1AD	NM_032325	0,71		0,94	0,02	2,32	1,86	1,44	0,87	**1,6**	0,82	0,21	2,8
JMJD6	NM_015167	1,14	0,24	1,06	0,29	2,56	0,04	1,56	0,03	**1,6**	0,85	0,01	3,0
HMOX1	NM_002133	1,27		0,60	0,18	2,58	0,11	1,57	0,48	**1,6**	1,02	0,09	2,5
MAFB	NM_005461	0,90		1,28	0,39	4,18	0,34	2,50	0,29	**1,7**	8,82	1,60	0,5
KCNJ2	NM_000891	0,85		1,35	0,52	2,62	0,43	1,55	0,31	**1,7**	2,61	0,90	1,0
FOS	NM_005252	1,11		2,26	0,23	70,19	4,29	41,08	4,19	**1,7**	44,85	10,47	1,6
ZNF10	NM_015394	0,87		0,77	0,03	3,75	0,61	2,18	0,32	**1,7**	1,39	0,11	2,7
FOSB	NM_006732	1,55		0,96	0,13	44,68	6,34	25,66	5,88	**1,7**	21,50	1,55	2,1
EIF2AK3	NM_004836	0,71		0,94	0,02	2,33	0,11	1,30	0,35	**1,8**	1,87	0,13	1,2
NR4A1	NM_002135	1,26	0,08	0,92	0,17	6,30	0,95	3,50	0,35	**1,8**	3,29	0,49	1,9
KLF6	NM_001300	1,26	0,34	0,79	0,29	2,14	0,06	1,19	0,02	**1,8**	1,50	0,08	1,4
DDIT4	NM_019058	0,42		1,07	0,09	2,35	0,27	1,29	0,15	**1,8**	1,26	0,06	1,9
NR4A3	NM_173198	0,76	0,34	1,35	0,47	6,75	0,23	3,62	0,42	**1,9**	5,12	1,09	1,3
CLU	NM_203339	1,13	0,07	0,82	0,21	2,26	0,28	1,21	0,06	**1,9**	1,15	0,02	2,0
DNAJB6	NM_058246	1,32	0,72	0,89	0,13	2,19	0,82	1,16	0,09	**1,9**	0,98	0,09	2,2
EGR1	NM_001964	1,28	0,26	0,83	0,15	232,11	72,57	121,01	24,99	**1,9**	127,76	17,65	1,8

Gene name	Systematic-Name	ave T4 vT0	sd	ave siHSF1 v siLUC T4	sd	ave pcDNA HS 6/ pcDNA NS	sd	ave K80Q+dox HS 6/ K80Q+dox NS	sd	fold inhibition HSF1-K80Q	ave dnHSF1+dox HS 6/ dnHSF1+dox NS	sd	fold inhibition dnHSF1
decreased in heat shocked HEK293 cells but not in heat shocked HeLa cells. None affected by K80Q													
GEMIN6	NM_024775	0,76		1,25	0,01	0,26	0,01	0,26	0,03	**1,0**	0,25	0,00	1,1
FLJ10213	NM_018029	0,91		0,93	0,07	0,27	0,08	0,37	0,00	**0,7**	0,45	0,04	0,6
MED7	NM_004270	1,13	0,00	0,93	0,09	0,28	0,06	0,32	0,07	**0,9**	0,33	0,02	0,8
HPS6	NM_024747	1,22		1,01	0,10	0,29	0,04	0,37	0,01	**0,8**	0,31	0,08	1,0
LOC151878	BC014063	1,05		0,94	0,00	0,30	0,09	0,33	0,01	**0,9**	0,31	0,05	1,0
WDR5B	NM_019069	0,71		0,95	0,10	0,30	0,01	0,35	0,09	**0,9**	0,36	0,12	0,8
ETAA1	NM_019002	0,96		1,28	0,34	0,30	0,00	0,27	0,06	**1,1**	0,32	0,05	0,9
C7orf68	NM_013332	0,80		1,02	0,04	0,31	0,02	0,32	0,01	**1,0**	0,22	0,01	1,5
GEMIN4	NM_015721	0,84	0,01	1,42	0,17	0,32	0,02	0,38	0,06	**0,9**	0,33	0,07	1,0
TMEM177	NM_030577	0,98		0,98	0,05	0,33	0,00	0,36	0,04	**0,9**	0,29	0,09	1,1
DDX47	NM_016355	0,97		1,32	0,03	0,35	0,02	0,32	0,07	**1,1**	0,44	0,02	0,8
MAT2A	NM_005911	0,86	0,14	1,41	0,44	0,35	0,04	0,32	0,02	**1,1**	0,29	0,05	1,2
LCMT2	NM_014793	0,94	0,01	1,12	0,09	0,36	0,01	0,39	0,09	**0,9**	0,39	0,05	0,9
C15orf39	NM_015492	1,47	0,41	1,29	0,24	0,38	0,04	0,40	0,03	**0,9**	0,43	0,14	0,9
C1orf156	NM_033418	1,31		0,89	0,10	0,38	0,01	0,46	0,02	**0,8**	0,43	0,03	0,9
C1GALT1C1	NM_152692	0,81		0,96	0,05	0,38	0,02	0,42	0,05	**0,9**	0,48	0,03	0,8
KIAA0754	NM_015038	0,98		0,93	0,02	0,39	0,06	0,44	0,05	**0,9**	0,43	0,11	0,9
XRCC2	NM_005431	0,84		1,01	0,26	0,40	0,03	0,44	0,02	**0,9**	0,55	0,06	0,7
CSTF1	NM_001324	0,81	0,02	1,22	0,16	0,40	0,02	0,42	0,09	**0,9**	0,37	0,07	1,1
MFAP1	NM_005926	0,85		1,15	0,05	0,40	0,00	0,47	0,06	**0,9**	0,50	0,08	0,8
KCNJ8	NM_004982	1,02	0,00	0,98	0,02	0,40	0,07	0,45	0,03	**0,9**	0,44	0,04	0,9
ZNF337	NM_015655	1,08	0,06	1,00	0,08	0,41	0,04	0,41	0,07	**1,0**	0,26	0,06	1,6
DGCR8	NM_022720	1,17	0,12	1,08	0,15	0,41	0,15	0,43	0,12	**1,0**	0,34	0,15	1,2
MTERF	NM_006980	1,16		1,04	0,14	0,43	0,04	0,47	0,15	**0,9**	0,42	0,06	1,0
FADD	NM_003824	0,77		1,15	0,17	0,43	0,05	0,43	0,07	**1,0**	0,31	0,07	1,4
ID4	NM_001546	1,07	0,13	0,97	0,07	0,43	0,01	0,45	0,01	**1,0**	0,42	0,07	1,0
TMEM187	NM_003492	1,03		0,85	0,03	0,43	0,04	0,48	0,10	**0,9**	0,64	0,16	0,7
MED4	NM_014166	0,82		1,14	0,13	0,44	0,06	0,47	0,03	**0,9**	0,53	0,04	0,8
TMPRSS5	NM_030770	1,22		0,82	0,05	0,44	0,02	0,60	0,11	**0,7**	0,49	0,08	0,9
GIN1	NM_017676	1,28		0,93	0,16	0,44	0,04	0,44	0,13	**1,0**	0,50	0,06	0,9
NAT1	NM_000662	0,73		1,14	0,13	0,45	0,01	0,54	0,03	**0,8**	0,46	0,01	1,0
IFIT1	NM_001548	1,86		0,46	0,22	0,45	0,02	0,53	0,05	**0,8**	0,46	0,02	1,0
ELK4	ENST00000357992	0,85	0,17	1,45	0,52	0,45	0,03	0,52	0,02	**0,9**	0,56	0,09	0,8
PRDX1	NM_002574	0,95		0,95	0,02	0,45	0,03	0,43	0,08	**1,0**	0,64	0,15	0,7
EZH1	NM_001991	0,90	0,08	1,04	0,07	0,45	0,04	0,52	0,05	**0,9**	0,49	0,01	0,9
OXTR	NM_000916	0,97		1,15	0,01	0,46	0,04	0,49	0,01	**0,9**	0,38	0,18	1,2
RWDD2A	NM_033411	1,00		0,81	0,14	0,47	0,01	0,48	0,08	**1,0**	0,40	0,09	1,2
XIST	NR_001564	1,05	0,03	0,95	0,04	0,47	0,09	0,44	0,04	**1,1**	0,23	0,01	2,1
BCL11B	NM_138576	0,99		1,01	0,12	0,49	0,04	0,53	0,07	**0,9**	0,40	0,02	1,2
SNRNP35	NM_022717	0,92		0,95	0,19	0,49	0,02	0,54	0,10	**0,9**	0,39	0,02	1,3
PTX3	NM_002852	1,60		0,69	0,05	0,49	0,03	0,49	0,02	**1,0**	0,82	0,07	0,6
DYRK3	NM_001004023	0,91		0,98	0,01	0,49	0,11	0,54	0,10	**0,9**	0,39	0,09	1,3
ZSWIM1	NM_080603	0,99		1,05	0,19	0,50	0,04	0,55	0,06	**0,9**	0,45	0,04	1,1

Chapter 8

Summary and discussion

Accumulation of abnormal proteins is associated with neurodegenerative syndromes such as Alzheimer disease, Parkinson disease and Huntington disease, all age-related diseases. The existence of these diseases emphasizes that defects impairing the body's ability to remove or repair damaged macromolecules effectively accelerates the aging process. Cells are equipped with several different categories of repair and surveillance: DNA surveillance and repair systems, RNA surveillance systems, anti-oxidant systems and systems like the heat shock response (HSR) and the unfolded protein response (UPR) that deal with proteotoxic stress. Deficiency of any of these cellular defence systems might tip the balance from repair to permanent damage. Impairment of any of these repair and surveillance systems will eventually lead to an increase in proteotoxic stress. Maintaining proteostasis during aging is expected to prevent or at least ameliorate age-related protein folding and inflammatory disease [1, 2].
Cells respond to cytoplasmic proteotoxic stress by producing additional chaperones. This response is called the heat shock response (HSR) and is mainly regulated at the level of transcription by heat shock factor 1 (HSF1). The activity of HSF1 declines with age. The protein is still present but can no longer be activated. One possible approach to prevent the decline in HSF1 activity during aging is either by targeting HSF1 directly or by targeting longevity related factors which control HSF1 activity [3]. One potential drawback of maintaining or increasing HSF1 activity is that HSF1 also increases the risk of cancer, also often an age-related disease [4]. The other known way to upregulate the stress system is to cause cellular stress, which ultimately may be deleterious and in aging cells this method will be less effective than in normal cells because of the lower activity of HSF1. An alternative is to maintain the capacity of the chaperoning network by boosting the expression of a single (co-)chaperone. To find ways to boost the defence and repair system without the deleterious effects, we need to know more about the system, its critical nodes and rate limiting steps.

HSF1 targets in stressed and non-stressed cells.
To understand the role of HSF1, and the consequences of the loss of activity thereof, better, we used cellular model systems based on transformed human embryonic kidney (HEK293) cells in which the transcriptional activity of HSF1 was inhibited by overexpression of HSF1 mutants. Dominant negative (dn)HSF1 lacks the transactivation domain but can still bind DNA. DnHSF1 is predicted to occupy the HSF1 binding sites and to repress transcription directed by those binding sites. HSF1-K80Q has lysine 80 in the DNA binding region replaced by glutamine, which inhibits DNA binding [3]. Expression of HSF1-K80Q should free the HSF1 binding sites (for an overview see figure 1). The rate of transcription of HSF1 target genes that are activated by HSF1 will decrease, while that of genes that are repressed by HSF1 will increase. The transcriptome changes as a result of exogenous expression of these

HSF1 mutants have been measured using microarrays (Chapter 4 and 7). Inhibiting HSF1 activity in the absence of stress by overexpressing dnHSF1 resulted in a downregulation of 10 genes, about half of which are canonical HSF1 target genes such as DNAJB1; no genes were upregulated. Inhibiting HSF1 activity in the absence of stress by overexpressing HSF1-K80Q resulted in a downregulation of 17 genes, while 11 genes were upregulated. There was no overlap between the 10 downregulated transcripts by dnHSF1 and the down- or upregulated transcripts in the HSF1-K80Q expressing cells. A comparison of our HSF1-K80Q microarray results with the published data using HeLa cells lacking HSF1 (siRNA) [5] showed only a single gene of which the transcript level changed significantly in non-stressed cells expressing HSF1-K80Q and in cells treated with siHSF1. The difference between the HEK293 and HeLa data could partly be explained by the fact that depletion of HSF1 by siRNA would free the chaperones which are usually complexed with HSF1 while the HSF1-K80Q mutant could capture more chaperones. Alternatively, HSF1 could participate in gene regulatory circuits for which the DNA binding activity is not required. To distinguish between these possibilities, the effect of lack of the HSF1 protein or just lack of HSF1 binding activity needs to be compared in the same cells. Still, the HSF1 dependency in the absence of stress of most of the genes found in our microarray analysis is probably tissue specific.

One of the strongest downregulated transcript in dnHSF1 expressing cells was PMVK (see Chapter 6). Further analysis of the PMVK promoter region showed the presence of an HSF1 binding site in the region encoding the 5'UTR. HSF1 is not required to maintain transcription of PMVK in either non-stressed or stressed cells as PMVK mRNA levels were not decreased in cells overexpressing HSF1-K80Q. These data suggest that HSF1 can regulate the PMVK promoter, but under which conditions HSF1 does so is still unknown.

Loss of regulation by HSF1 does have consequences for cellular robustness. The heat induced expression of several chaperones, like HSPA1A, DNAJB1 and HSPB1, is completely blocked in presence of dnHSF1 or HSF1-K80Q. A non-functional HSR prevents complete recovery from heat stress. Therefore, dnHSF1 or HSF1-K80Q expressing cells show a higher stress state 24 hours after heat stress than normal cells do. For example, 24 hours after heat shock the GADD34 and GADD45B mRNA levels are higher in cells expressing dnHSF1 or HSF1-K80Q compared with control cells. Evaluating the transcriptome changes in cells overexpressing the HSF1 mutants 24 hours after heat shock by microarray analysis might yield further insight in which stress pathways remain activated.

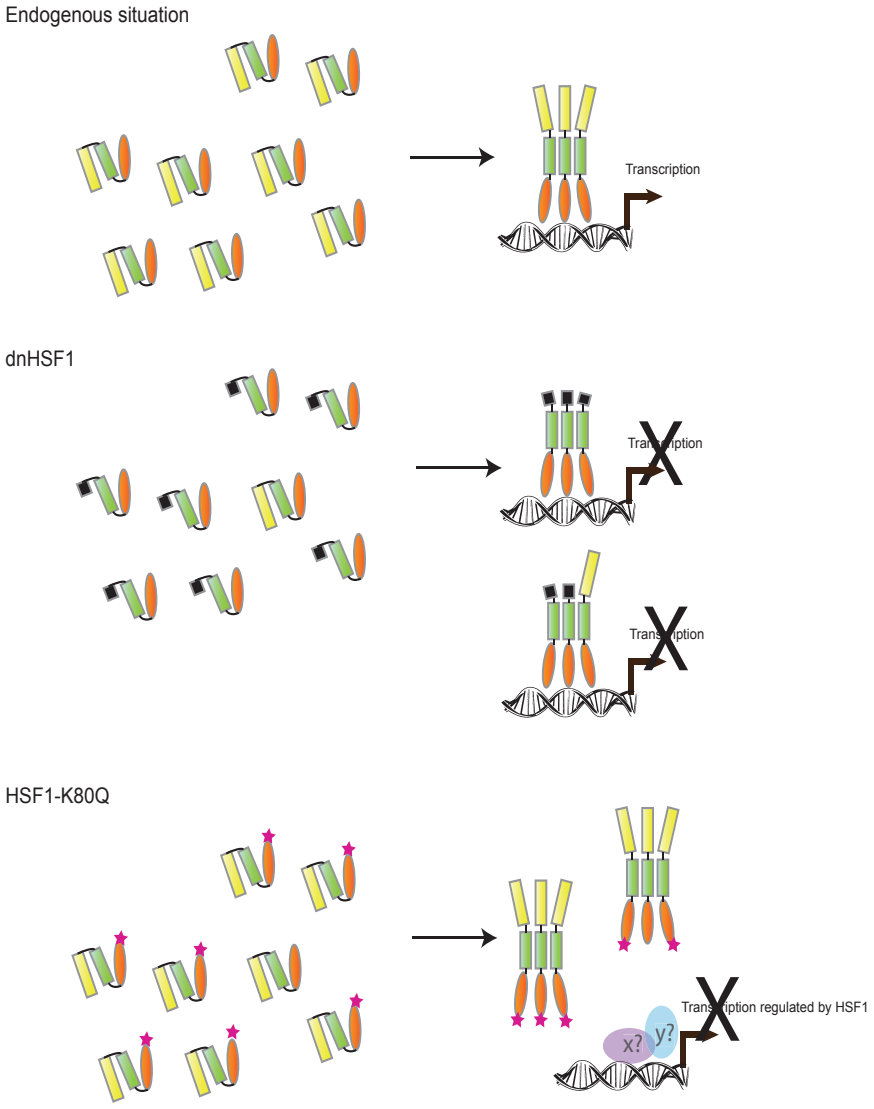

Figure 1 Cellular model systems used to inhibit HSF1 activity.
Dominant negative (dn)HSF1 lacks the transactivation domain but can still bind DNA. DnHSF1 is predicted to occupy the HSF1 binding sites and to repress transcription directed by those binding sites. HSF1-K80Q has lysine 80 in the DNA binding region replaced by glutamine, which inhibits DNA binding. Expression of HSF1-K80Q should free the HSF1 binding sites.

What are the critical nodes of the cytoplasmic chaperoning network?
Expression of dnHSF1 reduces the chaperoning capacity of the cell and HEK-dnHSF1 cells provide a good model to pinpoint critical nodes of the chaperoning network. A reduced chaperoning capacity results in the loss of the basal glucocorticoid response as well as a reduced refolding capacity in various organelles. We tested the loss of which chaperone was most critical for the inhibition of the glucocorticoid response. Since many gene products play a role at various stages of glucocorticoid receptor processing, it was very surprising that overexpression of individual proteins could rescue the negative effect of dnHSF1 on the glucocorticoid response. Nevertheless, the individual co-chaperones DNAJA1, DNAJB1 and ST13/Hip were able to rescue the dnHSF-mediated inhibition of the glucocorticoid response fully. Together these data show that the limiting node of chaperoning network is the Hsp70 folding machine, which is in turn is limited not by the level of Hsp70 itself, but rather by its co-chaperones (Chapter 4). These results are in apparent contrast with the results obtained when the chaperoning capacity of a cell is monitored by measuring refolding of heat-denatured luciferase. Exogenous expression of DNAJB1 did not restore refolding of luciferase, HSPA1A is required as well (with the exception of peroxisomal targeted luciferase) (Chapter 5). PolyQ aggregation inhibiting chaperones can still perform their action in cells that express the dominant negative HSF-1 construct [6, 7]. The main difference between the approach using the glucocorticoid response and the refolding assays is that the refolding assays were done in stressed cells, while the experiments using the glucocorticoid response or polyQ aggregation as a read out were performed using non stressed cells. The need for HSP70 will be higher during stress when more unfolded proteins are present. Which node of the chaperoning network is critical depends on the substrate tested and the compartment in which the substrate is located. Furthermore, the stress state of cells influences the need for particular chaperones. Increasing expression of (co-)chaperones to compensate for the loss of HSF1 regulated chaperones in aging cells thus needs to be tailored for specific substrates.

Does crosstalk between different stress responses exist?
The HSR and the UPR are both activated by proteotoxic stress, although in different compartments, and share cellular resources, such as the proteasome [8] and the eIF2α kinase regulatory pathway. eIF2α phosphorylation is a common response to different types of stress [9]. This means that ATF4 synthesis, which is enhanced by eIF2 α phosphorylation, is also common to various types of stress. The pattern of transcriptional activation by ATF4 is tailored to fit the type of stress and ATF4 targets are determined by stress specific factors [10]. The distinction between the transcriptional programs initiated by ATF4 as part of the different stress responses could be caused by differences in heteromeric partners. ATF4 produced by the UPR activates

some, but not all (see for example [10]), of the promoters also activated by the amino acid deprivation response. Although prototypical ATF4 target genes such as GADD34 and CHOP are upregulated during heat shock (see Chapter 2), ATF4 is not thought to play a significant role in the heat shock response. The HSR and the UPR also compete for resources in the, not unlikely, case that a stressor causes protein unfolding in both cellular compartments. How these resources are allocated when both responses are active is not known. Insight in possible crosstalk will help understanding the consequences of failure of these systems in (age-related) disease.

As already demonstrated by others [11], we observed that a heat shock evokes phosphorylation of eIF2α and splicing of XBP1 mRNA which are typical ER stress markers. Additionally, we show that spliced XBP1 protein (XBP1s) is synthesized and that transcription of constructs containing the consensus ERSE and UPRE elements is activated. These results together suggest that a typical UPR is activated upon heat shock. Nevertheless, identifying the transcription factors involved in the transcriptional activation of the UPR responsive promoters did not result in the expected set of transcription factors (See Chapter 2). Heat stressed HEK293 cells show HSF1 independent activation of the promoters of the genes for the ER resident chaperones HSPA5 and DNAJB9. The heat induced activation of the DNAJB9 promoter could not be blocked by inhibiting XBP1 splicing, and thus did not require spliced XBP1. Although inhibition of eIF2□ phosphorylation by overexpressing the C-treminal region of GADD34 inhibited the heat induced activation of the DNAJB9 promoter, the activation is not ATF4 dependent. The activation was also not ATF6 dependent, so another eIF2□ phosphorylation target than ATF4 is responsible for the heat stress induced activation of the DNAJB9 promoter. When the typical UPR promoters, the DNAJB9 and the HSPA5 promoters, become active in heat stressed cells, the heat shock induced UPR (XBP1 splicing and also eIF2□ phosphorylation) has already decayed. This possibly explains why another set of transcription factors needs to be recruited to activate these UPR promoters.

In heat stressed cells there is an effect on the activation of UPR genes. Does the activation of the UPR have an effect on typical heat shock genes? To answer this question we focused on XBP1s as we noted that XBP1s inhibits the activity of the HSPB1 promoter as well as that of the Drosophila melanogaster Hsp70 promoter after heat stress. Acosta Alvear et.al. [12] identified XBP1 binding sites in skeletal muscle from mice by performing a ChIP-on-chip assay. A number of the promoter regions binding XBP1 belong to HSF1 target genes such as BAG3 and SERPINH1. The heat stress induced transcriptional activation directed by either the heat shock element of BAG3 or the SERPINH1 promoter was also inhibited by spliced XBP1 and spliced XBP1 showed binding to the promoter regions of BAG3 and SERPINH1. However, spliced XBP1 actually enhanced the heat shock induced increase in BAG3 or SERPINH1 mRNA levels, while the HSPB1 mRNA and protein levels were reduced by XBP1s (Chapter 3). The responsiveness of canoni-

cal heat shock promoters to XBP1s and that of a canonical UPR promoter, DNAJB9, to heat stress suggest that there is crosstalk between the two stress systems.

Failure to maintain proteostasis is now generally accepted as one of the causes of aging.

Loss of regulation by HSF1 does have consequences for cellular robustness as a non-functional HSR prevents complete recovery from heat stress. Individuals differ in the rate and extent in which damage repair decreases during aging. Therefore, the likelihood of getting an age-related disease, such as the previously mentioned protein folding diseases but also chronic inflammatory diseases like arthritis differs. Deficiencies in the HSR change the transcriptome even in the absence of stress. In theory this would make it possible to select a set of genes which can function as diagnostic markers to determine the frailty of cells. Frailty is the inability of a cell to cope with stress. However, the HSF1 dependency in the absence of stress of most of the genes found in our microarray analysis was found to be tissue specific. More studies are necessary to find ubiquitous biomarkers to determine the frailty of cells. In addition to the genomic and proteomic approach one can also think of the metabolomic approach. Maybe the combination of the 'omics' could provide more answers. We know now that if cells turn out to have a defective HSR it is possible to compensate for the loss of HSF1 by increasing the expression of (co-)chaperones. It is important to tailor exactly which (co-)chaperones are needed: which (co-)chaperones are needed can differ for specific substrates, type of stressor and cell type also play a role.

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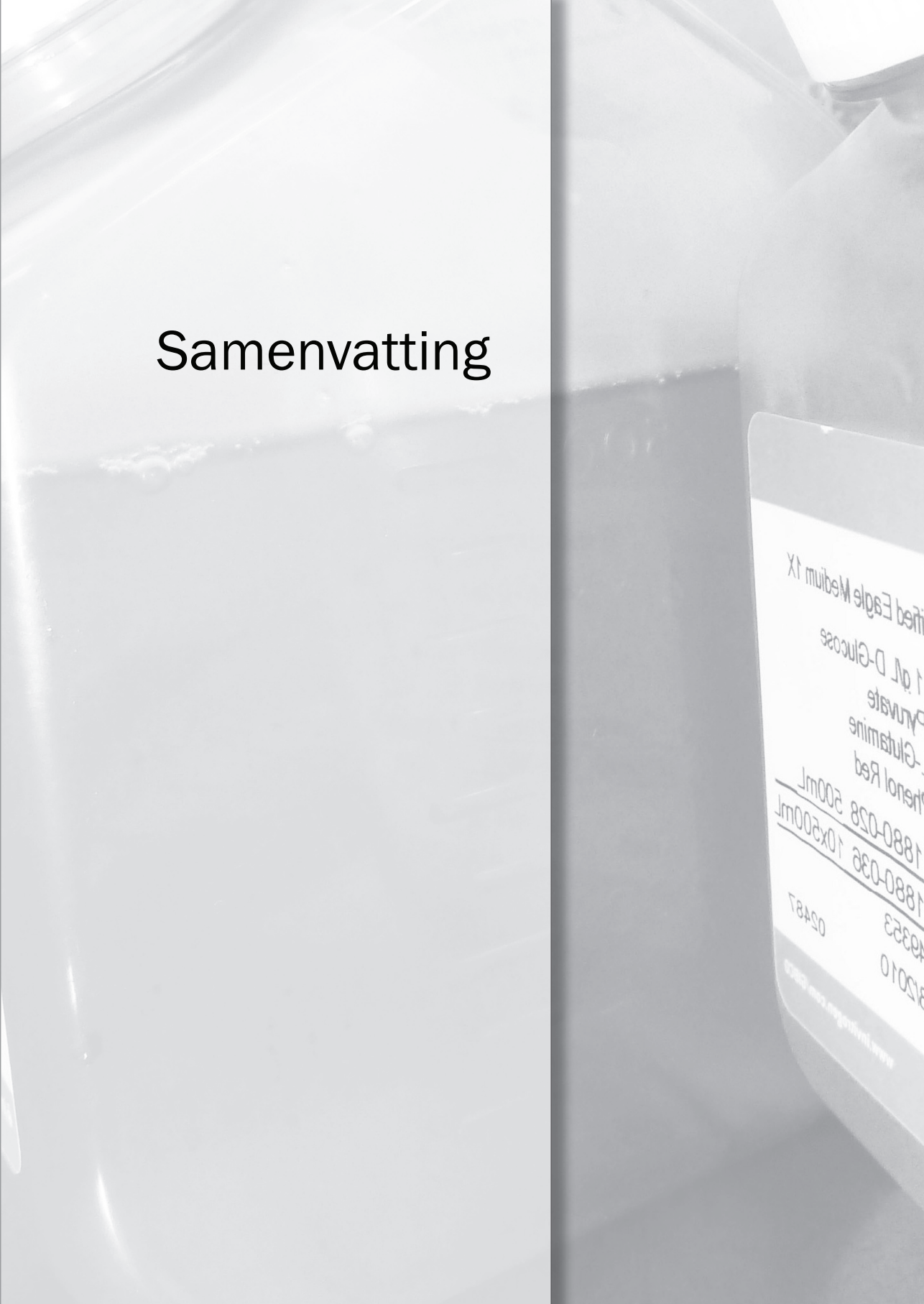
Samenvatting



Ons lichaam bevat heel veel eiwitten met allemaal verschillende taken en verantwoordelijkheden. De vorm van ieder eiwit ligt vast in de nucleotide volgorde van ons erfelijk materiaal: het DNA. Op dit DNA ligt de informatie van meer dan 20.000 genen. De nucleotide volgorde van deze genen wordt eerst afgelezen en omgezet naar een boodschapper (boodschapper RNA). Dit proces heet transcriptie en vindt plaats in de celkern. De boodschapper verhuist dan van de celkern naar het cytoplasma en daar wordt de informatie van deze boodschapper vertaald naar een aminozuurketen. Voordat deze aminozuurketen een functioneel eiwit kan zijn moet deze eerst gevouwen worden. Veel eiwitten worden in het cytoplasma gevouwen, maar membraan eiwitten en eiwitten die worden uitgescheiden door de cel worden in het endoplasmatisch reticulum (ER) gevouwen. Het vouwen van deze aminozuurketens gaat niet altijd goed. Ook kunnen verschillende stressoren (hoge temperaturen, UV straling, zware metalen, chemicaliën) eiwitten die een juiste vorm hebben zo beschadigen dat deze verkeerd gevouwen of geheel ontvouwen raken. Verder kunnen afwijkingen in de DNA volgorde die resulteren in een verandering van de aminozuur volgorde, ervoor zorgen dat sommige eiwitten sneller ontvouwen. De verkeerd gevouwen eiwitten zijn schadelijk voor de cel, omdat ze heel gevoelig zijn voor samenklontering. Deze klonten zijn giftig voor de cel omdat ze essentiële cellulaire processen verstoren (stress). Ontvouwen of verkeerd gevouwen eiwitten worden herkend door speciale (stress) eiwitten, de zogenaamde chaperonnes. Deze chaperonnes zorgen ervoor dat eiwitten weer juist gevouwen worden en hun functie kunnen blijven vervullen. Chaperonnes zorgen er ook voor dat eiwitten met onherstelbare schade worden afgebroken; zo wordt voorkomen dat deze eiwitten schade veroorzaken. Cellen zijn in staat om meer chaperonnes (waarvan er heel veel verschillende soorten zijn) te maken wanneer hier behoefte aan is. Een van de systemen dat hiervoor zorgt is de heat shock response (HSR) die wordt aangezet wanneer verkeerd gevouwen eiwitten opstapelen in het cytoplasma. De belangrijkste regelaar van de HSR is heat shock factor 1 (HSF1). Een ingewikkeld regelsytseem zorgt ervoor dat een cel weet wanneer een bepaald eiwit nodig is (bijvoorbeeld meer chaperonnes), dit wordt doorgegeven aan het gen en dan wordt er meer boodschapper gemaakt. Transcriptie factoren spelen hierbij een belangrijke rol. Transcriptie factoren binden aan een bepaalde DNA volgorde en activeren (of remmen) zo de transcriptie van een gen, waardoor er meer (of minder) boodschapper RNA van dat gen gemaakt wordt. HSF1 is een transcriptie factor, die de activiteit van verschillende genen, waaronder chaperonne genen, bepaalt.

Tijdens veroudering werkt de HSR die voor de aanmaak van chaperonnes zorgt steeds minder goed. Hierdoor neemt het herstelvermogen van de cel af en wordt de kans op verouderings gerelateerde eiwitstapelings-ziektes vergroot (zoals Alzheimer, Parkinson en de ziekte van Huntington). De HSR wordt geremd in een verouderde cel omdat de activiteit van HSF1 afneemt. HSF1 is nog steeds aanwezig in de cel, maar heeft problemen met het

binden aan DNA. Waarom dit gebeurt, is niet bekend. Een reden zou kunnen zijn dat verlaging van de hoeveelheid chaperonnes in een cel de kans op kanker verkleint. Chaperonnes kunnen namelijk ongeremde groei stimuleren en beschermend werken tegen celdood. Helaas heeft een gebrek aan chaperonnes ook nadelige consequenties, namelijk een gebrekkig herstelvermogen. Meer kennis over de rol van chaperonnes in het verouderingsproces kan het mogelijk maken om aftakeling van organismen te vertragen. Als het mogelijk is om de aanmaak van (een aantal) chaperonnes in een verouderde cel te herstellen, dan zou het herstelvermogen verbeterd worden. Ons onderzoek laat zien dat de aanmaak van enkele chaperonnes inderdaad al voldoende is om in sommige omstandigheden de negatieve effecten van een verminderde HSR op te heffen.

HSF1 afhankelijke boodschappers in normale cellen en cellen die zijn blootgesteld aan stress.

Wat gebeurt er in cellen die geen extra chaperonne eiwitten meer kunnen aanmaken in reactie op stress? Heeft HSF1 een rol wanneer er geen stress is? Dit zijn centrale vragen in dit proefschrift. Om de rol van HSF1 en het gevolg van minder actieve HSF1 beter te begrijpen, hebben we een cellulair model system ontwikkeld, waarvoor weefselkweek cellen (HEK293) gebruikt zijn. Deze cellen hebben extra coderende informatie gekregen zodat deze cellen een “fout” HSF1 (dnHSF1) kunnen maken. De “fout” in dnHSF1 houdt in dat dit eiwit heel erg op gewoon HSF1 lijkt en ook aan DNA kan binden, maar het uiteinde mist dat nodig is om de eigenlijke aanmaak van boodschappers voor verschillende eiwitten te stimuleren. HSF1-K80Q is een andere gemuteerde HSF1 en deze HSF1 heeft een “fout” in het gebied dat nodig is voor binding aan het DNA, waardoor deze HSF1 niet meer kan binden aan DNA. Aanmaak van HSF1-K80Q zorgt ervoor dat alle bindingsplaatsen waar HSF1 kan binden in het genoom geen HSF1 meer gebonden hebben, terwijl aanmaak van dnHSF1 als gevolg heeft dat de bindingsplaatsen wel bezet zijn maar het aflezen van de genen geblokkeerd wordt. De door HSF1 gestimuleerde aanmaak van boodschappers wordt geremd in aanwezigheid van dnHSF1 en HSF1-K80Q. In de literatuur is voorgesteld dat HSF1 niet alleen stimulerend maar ook remmend kan werken. Bij gebruik van HSF1-K80Q valt deze remming weg, terwijl bij dnHSF1 deze remming behouden blijft.

De hoeveelheid boodschappers van verschillende genen verschilt wanneer gewone cellen worden vergeleken met cellen die dnHSF1 of HSF1-K80Q hebben (hoofdstuk 4 en 7). Het remmen van HSF1 activiteit in de afwezigheid van stress met behulp van dnHSF1 resulteert in de remming van de aanmaak van 10 verschillende boodschappers, de aanmaak van de helft van deze boodschappers staat bekend als typisch HSF1 afhankelijk. Van geen enkele boodschapper wordt meer aangemaakt in cellen met dnHSF1. Wanneer HSF1 activiteit wordt geremd door aanwezigheid van HSF1-K80Q

wordt de aanmaak van 17 boodschappers geremd, terwijl de aanmaak van 11 boodschappers verhoogd is. Er is geen overlap tussen de 10 boodschappers waarvan de aanmaak wordt geremd door dnHSF1 en de 17 boodschappers waarvan de aan maak wordt geremd door HSF1-K80Q. Wanneer de in hoofdstuk 7 beschreven data verkregen met de HSF1-K80Q cellijn (zonder stress) wordt vergeleken met gepubliceerde data waarbij HeLa cellen zijn gebruikt die HSF1 missen (door gebruik van siRNA), zien we slechts één boodschapper waarvan de niveaus in zowel de HSF1-K80Q als in siHSF1 behandelde cellen lager zijn. Het verschil tussen HEK293-K80Q en HeLa-siHSF1 zou voor een deel verklaard kunnen worden als HSF1 kan participeren in de regulatie van boodschapper niveaus zonder dat DNA binding noodzakelijk is, maar waarschijnlijker is dat de HSF1 afhankelijkheid van het niveau van de verschillende boodschappers in de afwezigheid van stress sterk cel/weefsel specifiek is. Om erachter te komen wat hier precies aan de hand is moet het gebrek aan HSF1 of alleen het gebrek aan HSF1 DNA binding vergeleken worden in hetzelfde cel type.
In cellen met dnHSF1 zijn 10 verschillende boodschapper niveaus lager dan in normale cellen. Het niveau van de boodschapper van het gen PMVK wordt het sterkst geremd (zie ook hoofdstuk 6). Analyse van de nucleotide volgorde van het PMVK gen laat zien dat een bindingsplaats voor HSF1 is te vinden in het begin van het PMVK gen en niet, zoals gebruikelijk, voor het gen. Helaas weten we niet onder welke condities deze HSF1 bindingsplaats ook daadwerkelijk gebruikt wordt. De aanwezigheid van HSF1-K80Q beïnvloedt de hoeveelheid PMVK boodschapper niet en HSF1 is dus niet nodig voor de regulatie van de aanmaak van PMVK boodschapper in cellen met of zonder stress onder normale kweek condities. Mogelijk reguleert HSF1 de aanmaak van de PMVK boodschapper onder speciale omstandigheden of in sommige weefsels.
Wanneer HSF1 niet meer goed werkt, heeft dit gevolgen voor het herstelvermogen van een cel. De extra aanmaak van verschillende chaperonnes zoals HSPA1A, DNAJB1 en HSPB1 als gevolg van schade aangebracht door een hitte schok (tijdelijke verhoging van de omgevingstemperatuur) wordt volledig geremd door de aanwezigheid van dnHSF1 of HSF1-K80Q. Een niet functionele HSR kan volledig herstel na een hitte schok in de weg zitten. Wij zien dan ook dat cellen met dnHSF1 of HSF1-K80Q 24 uur na hitte schok nog verhoogde niveaus van bepaalde (niet HSF1 afhankelijke) stress boodschappers hebben, terwijl deze niveaus alweer gedaald zijn tot de normale niveaus in cellen die geen “fout” HSF1 hebben.

Wie zijn de belangrijkste spelers in het cytoplasmatische chaperonne netwerk?

Een cel heeft een uitgebreid chaperonne netwerk. Is het mogelijk om het herstelvermogen van een cel te verbeteren door de aanmaak van slechts een paar chaperonnes te verhogen? Ons onderzoek laat zien dat de aan-

maak van enkele chaperonnes al voldoende zou zijn om de negatieve effecten van een verminderde HSR op te heffen. Cellen met dnHSF1 hebben niet meer de mogelijkheid om cytoplasmatische chaperonnes aan te maken. Door deze lagere chaperonne capaciteit zijn de cellen met dnHSF1 minder gevoelig voor het corticosteroid hormoon. Wanneer echter extra DNAJA1, DNAJB1 of ST13/Hip (allemaal (co-)chaperonnes) wordt gegeven aan deze cellen dan herstelt de gevoeligheid voor hormonen weer (hoofdstuk 4). Ontvouwen eiwitten in cellen met dnHSF1 worden ook minder efficiënt hervouwen dan in normale cellen. Om dit te meten wordt het enzym luciferase, een eiwit, gebruikt, waarvan de activiteit heel makkelijk te meten is. Luciferase raakt snel ontvouwen als de omgevingstemperatuur verhoogd is en is dan niet meer actief. Wanneer de temperatuur weer normaal is, zorgen chaperonnes ervoor dat luciferase weer goed gevouwen wordt en de activiteit terug krijgt. Cellen met een gezonde chaperonne capaciteit kunnen luciferase weer snel hervouwen en de activiteit herstellen; cellen met dnHSF1 hebben een lagere hervouwingscapaciteit. Deze capaciteit kan verbeterd worden met extra HSPA1A. HSPA1A met extra DNAJB1 werkt nog beter, maar DNAJB1 alleen is niet effectief (hoofdstuk 5). Het grote verschil tussen het meten van corticosteroid hormoon gevoeligheid van de cellen en het meten van hervouwingscapaciteit zit in het feit dat bij de hervouwings assay stress wordt gebruikt en het chaperonne systeem dus belast wordt met ontvouwen eiwitten, terwijl dat bij de assay voor corticosteroid hormoon gevoeligheid niet het geval is. Welke speler in het chaperonne netwerk belangrijk is, is sterk afhankelijk van het substraat waarnaar gekeken wordt. Ook is de plaats in de cel waar een eiwit hervouwen moet worden van invloed en de hoeveelheid stress waar de cel op dat moment mee te maken heeft bepaalt ook welke chaperonnes vooral nodig zijn. Ons onderzoek laat zien dat de aanmaak van enkele chaperonnes al voldoende zou zijn om de negatieve effecten van een verminderde HSR op te heffen, maar er moet dan wel rekening worden gehouden met deze verschillende aspecten.

Communiceren verschillende stress systemen met elkaar?

De HSR, met als belangrijkste speler HSF1, wordt actief wanneer verkeerd gevouwen eiwitten opstapelen in het cytoplasma. Het ER heeft zijn eigen stress response en deze wordt de unfolded protein response (UPR) genoemd. Stapeling van verkeerd gevouwen eiwitten in het ER zorgt voor activering van drie verschillende paden, die ieder leiden tot aanmaak van een andere transcriptie factor, namelijk ATF4, ATF6 en XBP1. Deze drie transcriptie factoren zijn verantwoordelijk voor de aanmaak van boodschappers voor eiwitten die belangrijk zijn om de ER stress te verlichten (ER chaperonnes). De HSR en de UPR maken beide gebruik van verschillende hulpmiddelen in de cel, zoals de eiwit aanmaak- en afbraaksystemen. Wanneer een stressor ontvouwing van eiwitten in zowel het cytoplasma als in het ER veroorzaakt, dan zullen de HSR en de UPR moeten competeren voor de hulpbronnen die de cel te bieden heeft. Hoe deze hulpbronnen toegewezen

worden wanneer beide stress response systemen actief zijn is niet bekend. Inzicht in communicatie tussen de HSR en de UPR kan helpen bij het beter begrijpen van de consequenties die een gebrekkige HSR en/of een gebrekkige UPR hebben voor de ontwikkeling van (verouderings gerelateerde) aandoeningen.
We hebben aangetoond dat een hitte schok de aanmaak van ATF4 gang zet. Daarnaast wordt ook XBP1 gemaakt. Verder hebben we gezien dat ook het derde pad, dat zorgt voor de activering van ATF6, van de UPR wordt geactiveerd met hitte schok, al is deze activering mild in vergelijking met activering door specifieke ER stress. Deze resultaten duiden erop dat de drie paden van de UPR actief zijn na hitte schok. De activering van aanmaak van de boodschappers voor ER chaperonnes door een hitte schok gaat alleen net iets anders dan de activering van aanmaak van dezelfde boodschappers via typische ER stress. De aanmaak van DNAJB9 boodschapper, een ER chaperonne, wordt geactiveerd na hitte stress, maar de transcriptie factoren die nodig zijn om meer DNAJB9 boodschapper te maken zijn niet XBP1 en ATF4 zoals bij reguliere ER stress (hoofdstuk 2). Welke transcriptie factor na hitte schok wel nodig is, is nog niet bekend.
Wanneer extra XBP1 wordt gemaakt in een cel (wat ook gebeurt tijdens hitte schok), wordt de aanmaak van het HSPB1 boodschapper geremd; de activiteit van het HSPB1 gen wordt ook door HSF1 gecontroleerd. Hetzelfde geldt voor de activiteit van het Drosophila melanogaster Hsp70 gen: ook deze activiteit is afhankelijk van HSF1 en wordt geremd door XBP1s (hoofdstuk 3). Het feit dat de regulatie van twee genen, welke beide afhankelijk zijn van HSF1 voor activiteit, negatief wordt beïnvloed door XBP1s en het feit dat de aanmaak van de boodschapper van een ER chaperonne geactiveerd kan worden door hitte schok, suggereert dat de twee systemen met elkaar communiceren.

Een verouderde cel bevat minder chaperonnes en tijdens stress is deze niet in staat om genoeg extra chaperonnes bij te maken. De schade die ontstaat aan de eiwitten kan daardoor minder goed hersteld worden en beschadigde eiwitten worden minder goed afgebroken waardoor eiwitklontering optreedt. Vooral zenuwcellen zijn erg gevoelig doordat hun HSR bij voorbaat al minder efficiënt werkt. Vandaar dat veel verouderings gerelateerde stapelings ziekten neurologisch van aard zijn. Chaperonnes hebben ook het reguleren van het immuunsysteem als belangrijke taak. Wanneer de hoeveelheid chaperonnes in een cel afneemt neemt de kans op chronische ontstekingsziektes zoals reuma toe. Daarnaast heeft ieder mens een andere genetische achtergrond en dit wordt benadrukt wanneer de chaperonne capaciteit van een cel weg valt. Wanneer een genetisch verschil zorgt voor een iets andere eiwit volgorde die wat minder goed vouwt, dan kan dit defect ondervangen worden door chaperonnes en ontstaat er toch nog een functioneel eiwit. In een oudere cel gaat dat minder goed door een gebrek aan chaperonnes.

Doordat iedereen andere genetische variaties heeft zullen de defecten door ouderdom ook bij ieder individu anders zijn. Voor de behandeling van ouderdoms-gerelateerde ziektes zou het goed zijn om te weten wat de kwetsbaarheid van de cellen, i.e. het (on)vermogen tot schade herstel, is. Dit kan bepaald worden door te kijken naar de capaciteit van de HSR. Wanneer de HSR minder goed werkt heeft dit effect op het boodschapper patroon en op het eiwit patroon, zelfs zonder dat er stress aanwezig is. Helaas hebben onze data uitgewezen dat de HSF1 afhankelijkheid in de afwezigheid van stress sterk cel specifiek is. Wanneer blijkt dat de HSR minder goed werkt dan zou mogelijk de aanmaak van slechts enkele chaperonnes al voldoende zijn om de negatieve effecten van een beperkte HSR te verlichten. Daarom is het belangrijk dat er (synthetische of natuurlijke) stofjes worden gevonden die de aanmaak van bepaalde chaperonnes verhogen (op de plaats waar deze nodig zijn). Deze stofjes kunnen ingezet worden om de gevolgen van eiwitstapelings- of chronische ontstekingsziektes te verlichten. Uiteraard moet daarbij rekening worden gehouden met eventuele verhoogde kans op de ontwikkeling van kanker. Wanneer men chaperonnes wilt inzetten om de verschijnselen van ouderdoms-gerelateerde ziektes te verminderen moet er goed worden nagedacht over welke chaperonnes uit het hele netwerk er nodig zijn. Daarnaast is het wenselijk om deze chaperonnes alleen op de plaatsen waar zij nodig zijn te introduceren.

Dankwoord

Curriculum vitae

List of publications

Dankwoord

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Lieve Erik, ik kan me een leven zonder jou niet meer voorstellen. Bedankt voor het vele geduld dat je met mij en mijn gestress hebt (gehad). Ik kan je voor eindeloos veel dingen bedanken. Onder al die dingen vallen ook alle grote hoogtes die we samen bereikt hebben de afgelopen jaren. Geen berg of toren laat jij onbeklommen aan je voorbij gaan. De Eiffeltoren, Vogel, Oriental Pearl Tower, Petronas Twin Towers, Arthur's Seat (en dit is slechts een selectie). Laten we deze lijst nog veel langer maken!

Curriculum vitae

Lonneke Heldens werd geboren op 16 januari 1983 te Roosendaal. In 2001 behaalde zij het VWO diploma aan het Norbertuscollege te Roosendaal. In datzelfde jaar werd een begin gemaakt met de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. Tijdens haar studie liep zij stage bij de Immunotoxicologie groep van de afdeling Gezondheids Risico Analyse en Toxicologie (GRAT) te Maastricht. Onder begeleiding van Dr. Harald Moonen werkte zij daar aan een project getiteld: De effecten van PARP-1 remmers op de activatie van nuclear factor κB (NFκB) door immuunstimulerende stoffen. De mogelijke rol van PARP-1 remmers bij de preventie van chronische inflammatoire aandoeningen, zoals de longziekte COPD, stond bij deze stage centraal. Tijdens haar tweede stage bij de afdeling Moleculair Metabolisme en Endocrinologie (MME)/ Interne Geneeskunde aan de Universiteit Maastricht werkte zij, onder supervisie van Dr. Steven Meex en Dr. Carla van der Kallen, aan de identificatie van nieuwe genetische variaties die (mede)verantwoordelijk zijn voor de ontwikkeling van familiaire gecombineerde hyperlipidemie en diabetes mellitus type 2. In 2005 werd het doctoraal diploma behaald met als specialisatie Biologische Gezondheidskunde. In 2006 begon ze aan haar promotie-onderzoek aan de afdeling Biomoleculaire Chemie aan de Radboud Universiteit Nijmegen. Onder begeleiding van Prof. Dr. Lettie Lubsen resulteerde haar onderzoek uiteindelijk in dit proefschrift.

List of Publications

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Heldens L, Hensen SM, Onnekink C, van Genesen ST, Dirks RP, Lubsen NH. The unfolded protein response induced by a heat shock is not functional. PLoS ONE. 2011, 6 (8).

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Meex SJ, Weissglas-Volkov D, van der Kallen CJ, Thuerauf DJ, van Greevenbroek MM, Schalkwijk CG, Stehouwer CD, Feskens EJ, **Heldens L**, Ayoubi TA, Hofker MH, Wouters BG, Vlietinck R, Sinsheimer JS, Taskinen MR, Kuusisto J, Laakso M, de Bruin TW, Pajukanta P, Glembotski CC. The ATF6-Met[67]Val substitution is associated with increased plasma cholesterol levels. Arteriosclerosis, Thrombosis, and Vascular Biology. 2009, 29(9):1322-7.

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Appendix

Members of the families of heat shock proteins and their co-chaperones

Gene name	Acc. Nr.	Alternative name
HSF family		
HSF1	NM_005526.	
HSF2	NM_004506.	
HSF4	NM_001538.	
HSF5	NM_001080439	
HSFX1	NM_016153	HSFX2, LW-1
HSFY1	NM_033108	HSF2L, HSFY, HSFY2
HSPH family		
HSPH1	NM_006644	HSP105
HSPH2	NM_002154	HSPA4, APG-2, HSP110
HSPH3	NM_014278	HSPA4L, APG-1
HSPH4	NM_006389	HSP12A, hypoxia up-regulated 1
HSP90 family		
HSP90AA1	NM_005348	HSPC1, Hsp90α, LAP2, HSPCA
HSP90AB1	NM_007355	
HSP90B1	NM_003299	HSPC3, Hsp90β, HSPCB HSPC4, Grp94, TRA1, endoplasmin
TRAP1	NM_016292	HSPC5, HSP90L, TNF receptor-associated protein 1 (mitochondrial Hsp90)
HSPA (Hsp70) family		
HSPA1A/ B1	NM_005345	hsp72
HSPA1L	NM_005527	heat shock 70kDa protein 1-like
HSPA2	NM_021979	
HSPA5	NM_005347	GRP78, BiP
HSPA6	NM_002155	HSP70B'
HSPA8	NM_153201	HSC70
HSPA9	NM_004134	mortalin-2 (mitochondrial protein)
HSPA12A	NM_025015	
HSPA12B	NM_052970	KIAA0417
HSPA13	NM_006948	
HSPA14	NM_016299	STCH

Gene name	Acc. Nr.	Alternative name
DNAJ (Hsp40) family		
DNAJA1	NM_001539	DJ-2, HDJ2
DNAJA2	NM_005880	HIRIP4, Dnaj3
DNAJA3	NM_005147	Tid-1
DNAJA4	NM_018602	Dj-4, Hsj-4
DNAJB1	NM_006145	hsp40, HDJ1
DNAJB2	NM_006736	HSJ1
DNAJB3	NM_001001394	HSJ3
DNAJB4	NM_007034	Hsc40
DNAJB5	NM_012266	Hsc40, HSP40-3
DNAJB6	NM_005494	Mrj
DNAJB7	NM_145174	Dj5
DNAJB8	NM_153330	mDj6
DNAJB9	NM_012328	ERdj4
DNAJB11	NM_016306	ERdj3
DNAJB12	NM_001002762	Dj10
DNAJB13	NM_153614	Tsarg6
DNAJB14	NM_024920	FLJ14281
DNAJC1	NM_022365	ERdj1
DNAJC2	NM_014377	Zuotin related factor 1 (ZRF1)
DNAJC3	NM_006260	p58
DNAJC4	NM_005528	HSPf2
DNAJC5	NM_025219	cysteine string protein (CSP)
DNAJC5B	NM_033105	cysteine string protein beta (CSP-beta)
DNAJC5G	NM_173650	MGC107182
DNAJC6	NM_014787	Auxilin
DNAJC7	NM_003315	Ttc2
DNAJC8	NM_014280	spf31
DNAJC9	NM_015190	
DNAJC10	NM_018981	ERdj5
DNAJC11	NM_018198	
DNAJC12	NM_021800	jdp1
DNAJC13	NM_015268	
DNAJC14	NM_032364	
DNAJC15	NM_013238	
DNAJC16	NM_015291	
DNAJC17	NM_018163	
DNAJC18	NM_152686	
DNAJC19	NM_145261	
DNAJC20	NM_172002	J-type co-chaperone HSC20 (RP3-366L4.2)
DNAJC21	NM_194283	DnaJA5
DNAJC22	NM_024902	hypothetical protein FLJ13236
DNAJC23	NM_007214	SEC63
DNAJC24	NM_181706	ZCSL3
DNAJC25	NM_001015882	DnaJ-like protein
DNAJC26	NM_005255	cyclin G associated kinase (GAK), auxilin-2
DNAJC27	NM_016544	Ras-associated protein Rap1 (RBJ)
DNAJC28	NM_017833	C21orf55
DNAJC29	NM_014363	sacsin
DNAJC30	NM_032317	WBSCR18

Gene name	Acc. Nr.	Alternative name
HSPB (sHsp) family		
HSPB1	NM_001540	Hsp27
HSPB2	NM_001541	MKBP
HSPB3	NM_006308	HSPL27
HSPB4	NM_000394	αA-crystallin (CRYAA)
HSPB5	NM_001885	αB-crystallin (CRYAB)
HSPB6	NM_144617	HSP20
HSPB7	NM_014424	cvHSP
HSPB8	NM_014365	HSP22
HSPB9	NM_033194	FLJ27437
HSPB10	NM_024410	ODF1
Others		
HSPD1	NM_002156	HSP60, chaperonin
HSPE1	NM_002157	HSP10, chaperonin 10
SERPINH1	NM_001235	HSP47
CCT3	NM_005998	TCP1, subunit 3 (gamma)
Co-chaperones		
AHSA1	NM_012111	AHA1 homolog 1
AHSA2	NM_152392	AHA1 homolog 2
BAG1	NM_004323	
BAG2	NM_004282	
BAG3	NM_004281	
BAG4	NM_004874	
BAG5	NM_001015049	
PTGES3	NM_006601	p23
ST13	NM_003932	HIP
STIP1	NM_006819	HOP
STUB1	NM_005861	CHIP
AIP	NM_003977	
CDC37	NM_007065	
FKBP4	NM_002014	
FKBP5	NM_004117	
PPID	NM_005038	cyclophilin D
PPP5C	NM_006247	
SGTA	NM_003021	
TOMM70A	NM_014820	
TTC4	NM_004623	
UNC45A	NM_018671	